Proteolytic Activity of the ATP-dependent Protease HslVU Can Be Uncoupled from ATP Hydrolysis*

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HslVU is a new Escherichia coli ATP-dependent protease composed of two multimeric complexes: the HslU ATPase and the HslV peptidase. Prior studies indicated that HslVU requires ATP hydrolysis for the cleavage of peptides and proteins. We show here that ATP concentrations that activate hydrolysis of benzoylcarbonyl-Gly-Gly-Leu-7-amido-4-methylcoumarin are 50–100 fold lower than those necessary for degradation of proteins (e.g. casein). Also, the nonhydrolyzable analogs of ATP, 5'-adenylyl β,γ-imidodiphosphate (AMP-PNP) and adenosine 5'-[(β,γ-methylene)triphosphate, can support peptide hydrolysis, but only after an initial time lag not seen with ATP. This delay decreased at higher temperatures and with higher HslU or HslV concentrations and was eliminated by preincubation of HslU and HslV together. Thus, ATP hydrolysis accelerates the association of HslU and HslV, which occurs slowly with the nonhydrolyzable analog.

The addition of KCl stimulated 4–6-fold the peptidase activity with AMP-PNP present and eliminated the time lag, but KCl had no stimulatory effect with ATP. NH₄⁺ and Cs⁺ had similar effects as K⁺, but Na⁺ and Li⁺ were ineffective. AMP-PNP by itself supported hydrolysis of casein and other polypeptides only 20% as well as ATP, but in the presence of K⁺, Cs⁺, or NH₄⁺, AMP-PNP activated casein degradation even better than ATP, although it was not hydrolyzed. In addition, MgCl₂, MnCl₂, and CaCl₂ allowed some peptidase and caseinase activity in the absence of any nucleotide. However, Mn²⁺ and Ca²⁺, unlike Mg²⁺, abolished ATP hydrolysis and prevented further activation by ATP or AMP-PNP. These findings indicate that ATP binding to a high affinity site triggers the formation of an active state capable of peptide cleavage, although ATP hydrolysis facilitates this process. Rapid degradation of proteins requires a distinct state of the enzyme, which is normally reached through ATP hydrolysis at low affinity sites. However, AMP-PNP binding together with K⁺ can induce a form of HslVU that degrades proteins without energy consumption.

Protein breakdown in bacterial and eukaryotic cells requires ATP (1, 2). In the ubiquitin-proteasome pathway in eukaryotes, ATP hydrolysis is required for both substrate conjugation to ubiquitin and for the assembly and proteolytic activity of the 26 S proteasome, which consists of a 20 S proteolytic particle and a 19 S complex containing multiple ATPases (3, 4). In Escherichia coli and other bacteria, there are several multimeric endoproteases whose function is linked to ATP hydrolysis. The two best characterized enzymes of this group are the ATP-dependent proteases La (Lon) and ClpAP (Ti), which catalyze the rapid elimination of abnormal proteins and several short-lived normal regulatory proteins (1, 5).

Recently, we described the isolation of a new type of ATP-dependent protease in E. coli: HslVU (also called ClpQY), the product of the heat-inducible hslVU operon (6, 7). It consists of two distinct components: HslU, a 50-kDa protein with ATPase activity, and HslV, a 19-kDa protein that by itself has little or no peptidase activity but is activated markedly upon association with HslU. HslU is a member of the Hsp100/Clp family of ATPases that associate with the ClpP peptidase to form ATP-dependent proteases but can also function by themselves as molecular chaperones (8–10). HslV is of particular interest because it is homologous in sequence and tertiary structure to β-type subunits of the 20 S proteasome from eukaryotes, certain eubacteria, and archaeabacteria (11, 43). These proteases function by a novel mechanism, in which a conserved N-terminal threonine residue acts as the active site nucleophile in proteolytic activity (12, 13). HslVU appears to play a role in the rapid degradation of incomplete polypeptides induced by puromycin treatment and certain mutant polypeptides, which are stabilized in an hslV or hslU mutant (14, 15).

Since HslU and HslV could be co-immunoprecipitated from cell extracts in the presence of ATP, they appear to form a complex in vivo (6). Electron microscopic analysis indicates that HslU and HslV by themselves are both ring-like structures in which HslU behaves as a hexamer or heptamer (Mₑ 297,000 and 346,000) and HslV as two hexameric rings (Mₑ 228,000) (16, 17). The HslVU complex appears as a cylindrical four-ring particle in which two central HslV hexameric rings are flanked at both ends by an HslU ring (16). In certain respects, this structure resembles the ClpAP protease, which also forms a four-ring structure, with two heptameric rings (ClpP) sandwiched between a hexameric ring (ClpA) at each end (18). The HslVU protease thus has properties intermediate between those of the eukaryotic proteasome and the E. coli Clp protease. It also offers an attractive model system for studying the role of ATP hydrolysis in the mechanisms of the 26 S proteasome.

Studies of the E. coli La and ClpAP proteases have indicated several distinct functions for ATP hydrolysis in protein breakdown. ATP is necessary for the stability of La and the ClpA ATPase, and it promotes the assembly of the ClpA oligomer and facilitates its association with the ClpP peptidase (19, 20). It is noteworthy that ATP hydrolysis is also required for the assembly of the 26 S proteasome complex from the 19 S ATPase component and the 20 S proteasome particle, as well as for maintaining the stability of this large complex (4). In addition, ATP hydrolysis regulates proteolytic activity by activating the...
proteolytic site. In protease La, ATP binding allows formation of the peptidase site, and the subsequent hydrolysis of ATP temporarily inactivates it as part of an ordered reaction cycle (1). In the ClpAP protease, ATP binding to ClpA enlarges the active site on ClpP to allow cleavage of larger peptides (21). Both La and ClpAP exhibit a protein substrate-activated ATPase activity (20, 22). The hydrolysis of peptide bonds in protein substrates by La is proportional to the amount of ATP utilized; normally, two ATP molecules are consumed per peptide bond cleaved, although this stoichiometric relationship can be partially uncoupled (23). Degradation of ubiquitin-conjugated proteins by the 26 S proteasome also requires ATP hydrolysis, whose precise role is still unclear (4). Finally, ATP hydrolysis has been proposed to assist the unfolding of globular protein substrates and their translocation into the proteolytic chamber. Although cleavage of short peptides by proteases La and ClpAP occurs readily in the presence of nonhydrolyzable ATP analogs, the requirement for ATP hydrolysis seems to be more stringent with long polypeptides that may contain higher order structures (24, 25). In fact, ClpA and ClpX ATPases by themselves can function in a chaperone-like manner to disassemble multimeric proteins and in this way may help present them to the ClpP proteolytic component in an easily digestible form (8, 9). A proteolytically inactive form of the mitochondrial homolog of La has also been shown to exhibit a chaperone-like function (26). It seems likely that the 26 S ATPase possesses such chaperone-like activity as well, since only fully unfolded proteins may enter into the 20 S proteasome chamber (12).

ATP hydrolysis also plays a critical role in the function of the HslVU protease. Cleavage of ATP by the HslU ATPase dramatically activates hydrolysis of both short peptides and proteins by the HslV peptidase, while HslV stimulates ATP hydrolysis by HslU (6, 7, 41). Protein substrates (but not nondegraded proteins) also activate the ATPase activity of HslU (41). Thus, the proteolytic and ATPase functions of the HslVU protease appear tightly linked. Surprisingly, in contrast to all other known ATP-dependent proteases, the activity of HslVU against small peptides (for example, Z-Gly-Gly-Leu-Amc)1 was reported to require ATP hydrolysis, since very little activity was supported by the nonhydrolyzable ATP analog AMP-PNP (6).

We undertook these studies to examine more closely the role of ATP binding and hydrolysis in HslVU function. We demonstrate here conditions that can eliminate the requirement for nucleotide hydrolysis for degradation of peptides and proteins. These studies also indicate the existence of multiple functional states of HslVU that are normally achieved by ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Materials—Purified HslV and HslU were prepared as described previously (16). Benzylocarbonyl-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-Gly-Gly-Leu-Amc) was obtained from BACHEM (Switzerland), and 10 mM stocks were prepared in 100% Me2SO. Bovine β-casein and α-lactalbumin were obtained from Sigma. 14C-Labeled β-casein was prepared by reductive methylation (28) and stored in 20 mM Bis-Tris propane-HCl (pH 7.8). The activity was monitored by the amount of newly generated γ-amino groups that reacted with fluorescamine, as described previously (35). Usually, 10 times more enzyme was used in measuring protein breakdown than for assays of peptide hydrolysis.

ATPase Assays—Phosphate production under conditions used for casein hydrolysis was measured by the nonradioactive malachite-molybdate method as described before (29).

Other Assays—Protein concentrations were determined by the method of Bradford (30) and confirmed by measuring the absorbance at 280 nm.

RESULTS

ATP Concentrations Needed for Peptide and Protein Degradation—The HslVU protease has been shown to hydrolyze the fluorogenic peptide Z-Gly-Gly-Leu-Amc and a number of polypeptide substrates in the presence of ATP (6, 7, 41). To determine the ATP concentrations required for these activities, purified HslV and HslU were combined and incubated with this fluorogenic tripeptide or 14C-labeled casein and different concentrations of ATP. As shown in Fig. 1, the Km value for ATP was 3–4 μM for peptide hydrolysis but 200–300 μM for 14C-labeled casein degradation. Hydrolysis of two other polypeptides, α-lactalbumin and IGF-1, showed similar Km values for ATP as casein hydrolysis (data not shown). Interestingly, prior studies indicated that the Km for ATP hydrolysis by HslVU was ~300 μM (7), which resembles the Km values for protein degradation, but was 100-fold higher than that for peptide hydrolysis. Since the concentration of ATP needed to activate peptide hydrolysis was much lower than that for protein degradation, there seem

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1 The abbreviations used are: Z, carboxbenzoxacy; Amc, 7-amino-4-methylcoumarin; AMP-PNP, 5′-adenylyladenyl β,γ-imidodiphosphate; AMP-CP, adenosine 5′-(β,γ-methylene)triphosphate; ATP-γ-S, adenosine 5′-(β,γ-thiotriphosphate); IGF-1, insulin-like growth factor 1.
to be two distinct active states of HslVU; ATP at a high affinity site can activate cleavage of small peptides, while its occupancy of a distinct, low affinity site is essential for hydrolysis of proteins.

Nonhydrolyzable ATP Analogos Support Peptidase Hydrolysis after a Time Lag—We undertook a systematic study on the ability of various nucleotides to stimulate peptidase activity by HslVU. As reported previously (6), little or no peptide cleavage occurred in the absence of ATP or in the presence of ADP, AMP, CTP, GTP, UTP, or the nonhydrolyzable ATP analog AMP-PCP. However, in contrast to prior reports, we found that the nonhydrolyzable analogs AMP-PNP and AMP-CPP supported significant peptidase hydrolysis (Table I, control). (No meaningful results could be obtained with ATPγS because the commercially available ATPγS was contaminated with appreciable amounts of ATP.)

One major difference was found between the peptidase activity seen with ATP and that with the nonhydrolyzable analogs; ATP addition immediately activated peptidase hydrolysis, while AMP-PNP (and AMP-CPP) allowed peptide cleavage only after an initial delay (Fig. 2). In previous studies with these nucleotides (6, 7), peptidase activity had been determined only at a single time point (usually 10 or 15 min), which was during or just after this time lag. Therefore, it was concluded that nonhydrolyzable ATP analogs could not support significant peptide hydrolysis. For example, in a typical experiment (Fig. 2), 10 min after nucleotide addition peptide cleavage with AMP-PNP was about 14% of that with ATP, but after the lag period, the rate of peptide cleavage in the presence of AMP-PNP was linear and approximately 40% of the initial rate seen with ATP (0.22 nmol/min and 0.60 nmol/min, respectively).

These findings indicate that HslVU activation occurs much more rapidly with ATP present than with the nonhydrolyzable analogs. Because HslU oligomerization requires nucleotide binding (16, 31), one component of enzyme activation may be HslU self-assembly, although ADP can also support this process, but it does not allow peptide hydrolysis. In addition, since both HslU and HslV must be present for rapid peptide hydrolysis, ATP may facilitate the association between HslU and HslV. If so, conditions that favor complex formation should decrease the lag time seen with AMP-PNP. As shown in Fig. 3A, the lag time preceding peptide hydrolysis decreased with increasing amounts of HslU. Thus, the slow step in peptide hydrolysis with AMP-PNP may be HslU self-assembly. However, the lag time also decreased with increasing amounts of HslV when HslU concentration was held constant (Fig. 3B).

Therefore, the slow activation with AMP-PNP appears at least in part attributable to the time required for the association between HslU and HslV. In addition, the magnitude of the lag time with AMP-PNP depended on the temperature. Lowering the temperature from 37 to 30 or 20 °C extended the lag time 2–3-fold (Fig. 3C). To test if self-assembly of HslU oligomers and formation of the HslVU complex may contribute to this delay preceding enzyme activation in the presence of nonhydrolyzable analogs, these components were preincubated with AMP-PNP before substrate addition. Preincubation of HslU with AMP-PNP for 5 min at 37 °C reduced partially the initial lag and peptidase activity with AMP-PNP together with AMP-PNP completely eliminated it (Fig. 3D). As expected, preincubation of HslV with the nucleotide did not affect the lag.

In the presence of ATP, the extent of the stimulation of the HslV peptidase and proteinase activity depends on the concentration of HslU (7, 41). For protein degradation, the maximal activity was obtained when the molar ratio of HslU rings to HslV rings reached 1:1 (a mass ratio of 3:1) (41). Fig. 4 shows that a similar ratio of HslU to HslV also gave the maximal peptidase activity with ATP. In the presence of AMP-PNP, the level of peptidase activity after the lag also depended on the amount of HslU. At all HslU concentrations tested, the rate of peptide hydrolysis with AMP-PNP (after the lag) was less than with ATP, but the difference between the activity with ATP and AMP-PNP diminished with increasing HslU (Fig. 4). Perhaps the simplest explanation for these findings is that HslU self-assembly and association with HslV are less efficient with AMP-PNP than with ATP. Therefore, in the presence of AMP-PNP, higher concentrations of HslU are needed to achieve the same level of activity as with ATP.

FIG. 2. The nonhydrolyzable analog AMP-PNP, unlike ATP, activates hydrolysis of Z-Gly-Gly-Leu-Amc after a time lag. Peptidase hydrolysis was assayed with 1.5 μg/ml HslV and 4.3 μg/ml HslU for different times after the addition of the nucleotide.

### Table I

| Addition (1 mM) | No KCl | 50 mM KCl |
|----------------|--------|-----------|
| None           | 8.0    | 5.5       |
| ATP            | 100    | 75        |
| AMP-PNP        | 37     | 141       |
| AMP-CPP        | 40     | 158       |
| AMP-PCP        | 5.8    | 5.1       |
| ADP            | 4.9    | 3.4       |
| AMP            | 8.3    | 5.2       |
| CTP            | 2.3    | 5.1       |
| GTP            | 1.6    | 1.7       |
| UTP            | 3.4    | 3.6       |

KCl stimulates the hydrolysis of Z-Gly-Gly-Leu-Amc in the presence of AMP-PNP and AMP-CPP.

Reaction mixtures contained 1.5 μg/ml HslV and 4.3 μg/ml HslU. For incubations with ATP, AMP-PNP, and AMP-CPP, assays were performed for various periods up to 40 min. For incubations with the remaining nucleotides, activity was assayed at 40 min. The rate of peptide hydrolysis using ATP without KCl was expressed as 100%.
4–5-fold peptide hydrolysis with AMP-PNP present, such that this activity was similar to or greater than that with ATP. The addition of KCl also eliminated the time lag preceding peptide cleavage with AMP-PNP alone. When the ATP analog was not present, KCl did not stimulate peptide hydrolysis by HslV or by HslVU. The maximal stimulation was reached at 100–200 mM KCl, which resembles the intracellular concentrations of KCl in E. coli (32). The concentration of KCl for half-maximal peptidase activity was approximately 20 mM (Fig. 6A). A similar stimulation was seen when KCl was replaced by potassium acetate (data not shown); thus, only the cation seems critical.

While $K^+$ increased the $V_{\text{max}}$ of peptide hydrolysis in the presence of AMP-PNP, it did not alter the enzyme's affinity for the nucleotide ($K_{\text{act}} \sim 300 \, \mu\text{M},$ Fig. 6B). The concentration of AMP-PNP needed to activate peptidase function was thus much higher than that of ATP ($\sim 300 \, \mu\text{M}$ versus $\sim 4 \, \mu\text{M}$). KCl failed to enhance peptidase activity in the presence of various other nucleotides including ADP, AMP, CTP, GTP, UTP, and AMP-PCP (Table I). Only the effects of AMP-PNP and AMP-CPP, which by themselves allowed peptide cleavage after a time lag, were stimulated by $K^+$.

$K^+$ Enhances the Effect of Nonhydrolyzable ATP Analogs on Protein Degradation—Because of these unusual effects of AMP-PNP and $K^+$ on peptide hydrolysis, we tested whether they could support the proteinase activity by HslVU. AMP-PNP by itself allowed some hydrolysis of $^{14}\text{C}$-labeled $\beta$-casein into acid-soluble products, at approximately 20% of the rate with ATP. No clear time lag was demonstrable (although such a delay might not be evident because of the low proteinase activity with AMP-PNP). As found with the peptide substrate,
the addition of K\(^+\) greatly activated casein degradation in the presence of AMP-PNP, although K\(^+\) by itself did not stimulate proteolysis (Fig. 7). In fact, the caseinase activity attained with K\(^+\) and AMP-PNP was consistently higher than that with ATP. K\(^+\) and AMP-PNP were also observed to stimulate hydrolysis of \(\alpha\)-lactalbumin and IGF-1 to a similar extent (data not shown). The addition of K\(^+\) not only did not stimulate protein degradation in the presence of ATP, but it even reduced this activity by about 30\%, and under these conditions it did not affect the rate of hydrolysis of ATP (Fig. 8).

The concentration of AMP-PNP needed for activating casein degradation was similar to that for peptide hydrolysis (\(K_{\text{act}} \approx 300 \mu\text{M}\)) with or without KCl (Fig. 6B, and data not shown). We also tested the possibility that K\(^+\) somehow allowed hydrolysis of the ATP analog AMP-PNP. However, no phosphate release was detected with AMP-PNP, with or without KCl present (Fig. 8). This finding also ruled out the remote possibility that trace amounts of ATP contaminated the AMP-PNP stock and were being hydrolyzed under these conditions. As was found for peptidase activity (Table I), KCl enhanced casein degradation with AMP-PNP and AMP-CPP but not with other nucleotides (data not shown).

Certain Other Monovalent Cations Influence Proteolytic Activity Like K\(^+\)—These findings clearly indicate that ATP binding together with K\(^+\) can eliminate the need for ATP hydrolysis in activating HslVU for peptide and protein hydrolysis. We therefore undertook a more systematic examination of the effect of various other monovalent cations. At 50 mM, all monovalent ions tested inhibited to varying degrees peptidase activity (Table II). The extent of this stimulation increased further (by 15–20\%) when KCl, NH\(_4\)Cl, and CsCl concentrations were raised up to 200 mM (Fig. 6A, and data not shown). On the other hand, Na\(^+\) and Li\(^+\) consistently had no stimulatory effect.

As found with peptidase activity, none of these ions tested stimulated casein hydrolysis in the presence of ATP, but K\(^+\), NH\(_4\)\(^+\), and Cs\(^+\) dramatically enhanced the low rate of casein hydrolysis seen with AMP-PNP (Table II) and did not allow hydrolysis of this analog (Fig. 8, and data not shown). Since these ions had very similar effects on the degradation of a short peptide or a large protein, they probably influence conformation of the enzyme rather than conformation of the polypeptide substrate.

Effect of Divalent Cations on HslVU Peptide and Protein Hydrolysis—Prior studies showed that the ATP-dependent peptidase activity of HslVU was completely abolished in the presence of EDTA, a chelator of divalent cations (6). Since most ATP-dependent reactions require Mg\(^{2+}\) or Mn\(^{2+}\) as a cofactor, we characterized further this requirement for divalent cations. At 5 mM, Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\) all allowed some peptidase and caseinase activity, even in the absence of any nucleotide (Table III), as had been found with protease La (22). Under these conditions Mn\(^{2+}\), which was more effective in activating peptide and casein hydrolysis than Mg\(^{2+}\) or Ca\(^{2+}\), stimulated activity to 25–30\% of that with Mg-ATP. However, in the presence of Mn\(^{2+}\), the addition of ATP or AMP-PNP did not result in a further stimulation of proteolytic activity, in contrast to Mg\(^{2+}\). With Ca\(^{2+}\), the addition of nucleotide also did not stimulate the caseinase activity, and it even reduced the peptidase activity. Also in contrast to Mg\(^{2+}\), neither Mn\(^{2+}\) nor Ca\(^{2+}\) could support ATP hydrolysis (Table III). The effect of these divalent cations requires the presence of HslVU, since no stimulation of activity was seen with HslVI alone (data not shown). Somewhat higher peptidase activity was obtained when the concentrations of MgCl\(_2\), MnCl\(_2\), and CaCl\(_2\) were raised to 20 mM (data not shown). Thus, all of these divalent cations can partially enhance the activity of HslVU and do so independently of nucleotide binding or hydrolysis, but the activation by ATP or AMP-PNP specifically requires Mg\(^{2+}\) as a cofactor.

DISCUSSION

Although prior studies had indicated that the proteolytic activity of HslVU was tightly linked to ATP hydrolysis (6, 7), the present results demonstrate that nucleotide binding without hydrolysis is sufficient to support peptide cleavage after a lag time and that nonhydrolyzable ATP analogs in the presence of KCl can even support maximal rates of degradation of both peptides and proteins. Such a complete uncoupling of protein degradation from ATP cleavage has not been found previously with any other known ATP-dependent protease. For example, with nonhydrolyzable analogs, protease La can cleave short peptides as rapidly as with ATP, but it does not degrade casein or other proteins in a processive manner, as occurs with ATP (22). With AMP-PNP, La can make limited cleavages in casein (33) and can degrade slowly some short, unfolded polypeptides (25). The ClpAP protease does not require a nucleotide for cleavage of very small peptides by the ClpP component, but AMP-PNP binding to the ClpA ATPase alters the active site such that larger peptides can be cleaved (21), and ATP hydrolysis is necessary for processive degradation of larger polypeptides, such as casein (24). Degradation of the E. coli heat-shock transcription factor σ\(^{32}\), catalyzed by the membrane-bound protease FtsH (HflB), does not occur without ATP hydrolysis (34). The eukaryotic 26 S proteasome also requires ATP hydrolysis to degrade ubiquitin-conjugated proteins, although it can degrade certain nonubiquitinated proteins, including casein, without ATP hydrolysis (4). This ability of K\(^+\) to function with nonhydrolyzable analogs seems specific for the HslVU protease and was not seen with ClpAP or 26 S proteasome.\(^2\)

Presumably, HslVU normally functions in vivo through a cyclical mechanism in which ATP binding allows the formation of an active form, but ATP hydrolysis to ADP must at least

\(^2\) H.-C. Huang, K. M. Woo, and A. L. Goldberg, unpublished observations.
partially inactivate the enzyme until a new ATP is bound. ADP has been shown to inhibit proteolysis by HslVU (6) and is also a potent inhibitor of protease La (1). This enzyme is normally maintained in an inactive state by tightly bound ADP, until the binding of a protein substrate triggers ADP release and binding of a new ATP, which leads to activation of proteolysis. *In vivo*, such cycles of protease activation and inactivation following ATP hydrolysis may provide an important mechanism that helps to prevent excessive or nonselective degradation of cell proteins. While ATP hydrolysis by HslVU transiently induces an active state that can digest both peptides and proteins, AMP-PNP binding with $K_{\text{act}}$ appears to induce a similar active conformation for a prolonged period. This activation process clearly can occur without ATP hydrolysis.

The present findings indicate that ATP serves at least two distinct roles in a multiple-step process leading to HslVU-catalyzed proteolysis: 1) it allows the formation of an active form sufficient for peptide hydrolysis, and 2) it induces additional conformational changes necessary for protein hydrolysis. This conclusion is based on our finding of the two distinct $K_{\text{act}}$ values for ATP, and related observations of Chung and co-workers (7, 41). While only 3 or 4 $\mu$m ATP was sufficient to maximally activate peptide cleavage (Fig. 1), both protein hydrolysis and ATPase activity (Fig. 1, Refs. 7 and 41) required 50–100-fold higher concentrations of ATP. Thus, ATP binding at a high affinity site triggers formation of a state fully active against tripeptides, while binding of additional ATPs at low affinity site(s) is necessary for catalyzing protein degradation and for maximal ATP hydrolysis. The degradation of a protein substrate must involve a number of steps, e.g., the binding of the protein to HslVU, processive movement of the substrate to additional active sites in the HslV central chamber, and the release of peptide products. Since the binding of AMP-PNP in the presence of $K^+$ seems to mimic the most active form of the enzyme, which presumably is induced transiently by ATP before its hydrolysis to ADP, protein breakdown by HslVU can occur without the enzyme transiently cycling through an inactive conformation with bound ADP.

Although ATP hydrolysis is not essential for peptide hydrolysis, the formation of the active state is clearly facilitated by ATP hydrolysis. In the presence of a nonhydrolyzable analog, peptidase activity was evident only after a time lag, which was...
longer at lower temperatures. The present studies indicate that the major factor contributing to this lag period is the time necessary for association of the components of HslVU into an active form. For activity, HslU subunits must assemble into the active heptameric (or hexameric) rings. This process requires the presence of ATP, ADP, or an ATP analog (16), and a mutation in the ATP-binding site prevents HslU oligomerization (31). Presumably, ATP hydrolysis (or the addition of K+ with AMP-PNP) promotes this oligomerization process much better than the nonhydrolyzed analog alone. Accordingly, pre-incubation of HslU with AMP-PNP before the addition of HslV and the peptide substrate reduced the time lag. In addition, the lag in peptidase activity may be due to the time required for the association between HslV and HslU oligomers, and ATP hydrolysis may accelerate this process also. Since increasing the concentration of either HslU or HslV reduced the time lag in the presence of AMP-PNP and preincubation of both components together completely eliminated it, ATP hydrolysis must facilitate the formation of active HslVU complex, which occurs slowly with nonhydrolyzable analogs in the absence of K+. For the other two-component ATP-dependent proteases, ATP hydrolysis promotes the association between the proteolytic and the ATPase component (4, 20). Nonhydrolyzable analogs can support complex formation between ClpA and ClpP but not nearly as well as ATP (20). Similarly, formation of the eukaryotic 26 S proteasome complex from its 19 and 20 S components requires ATP hydrolysis (4). In fact, assembly of these complexes may be a way that ATP hydrolysis regulates the rate of protein breakdown in cells.

A hallmark of the ATP-dependent proteases La and ClpAP is their ability to degrade proteins in a highly processive manner, i.e., they cleave proteins to small peptides without the release of polypeptide intermediates (1). ATP hydrolysis was shown to be necessary for this processive mechanism of La (33), but more recent studies have suggested that processive breakdown of small polypeptides may occur without energy consumption (25). Also, the 20 S proteasome from archaeabacteria is able to degrade an unfolded protein in a highly processive manner without ATP (35). In related studies we have found that HslVU also operates via a processive mechanism and that either ATP or AMP-PNP with K+ allows processive degradation of proteins.

One possible function for the ATP hydrolyzed during protein degradation by the proteasome or related enzymes is to facilitate the unfolding of proteins into an extended conformation capable of entering into the complex's proteolytic core, e.g., into the HslV dodecamer. Both the ClpA and ClpX ATPases have the "chaperone-like" ability to disassemble certain protein complexes, and this function may aid in the digestion of protein substrates (8, 9). It seems likely that HslU may also possess such an ATP-dependent dissociating or unfold function.

However, since casein degradation can proceed in the presence of AMP-PNP and K+, energy consumption from ATP hydrolysis is clearly not necessary for unfolding this substrate. It should be noted that casein and the other substrates studied here (reduced and denatured α-lactalbumin and IGF-1) have little secondary or tertiary structure. Possibly with more structured proteins as substrates, ATP hydrolysis and chaperone-like activity of HslU may be necessary for their degradation.

The ability of K+, NH4+, and Cs+ to activate HslVU in the presence of a nonhydrolyzable ATP analog suggests that these monovalent cations may help influence the nucleotide binding site (or the enzyme's conformation) in a similar manner as is achieved transiently during ATP hydrolysis. A requirement for these same ions has been found for other enzymes catalyzing phosphoryl transfer reactions such as pyruvate kinase (36). For enzymes of this family as for HslVU, Na+ and Li+ could not substitute for K+. Interestingly, a role for K+ has also been described in the mechanism of the ATP-dependent molecular chaperones Hsp70 and GroEL (37, 38, 42). ATP binding to Hsp70 triggers the release of a protein substrate from the chaperone, and this process has an absolute requirement for K+ (37). A recent report has confirmed this requirement for K+ (or NH4+) for Hsp70 function (39). This ion is also required for GroEL-assisted protein folding in vitro and apparently increases the affinity of GroEL for ATP (38). By contrast, with HslVU, K+ did not increase the apparent affinity (Kd) for AMP-PNP. Thus, the monovalent ion probably acts after nucleotide binding to facilitate the transition to the active state. Possibly, K+ and nucleotides bind at separate sites, but both must be occupied for the formation of the most active state of the enzyme.

3 H.-C. Huang and A.L. Goldberg, unpublished results.
**ATP Hydrolysis and the HslVU Protease**

**Table II**

| Addition (50 mM) | Hydrolysis of Z-Gly-Gly-Leu-Amc | Hydrolysis of 14C-Casein |
|------------------|---------------------------------|-------------------------|
|                  | ATP | AMP-PNP | Ratio | ATP | AMP-PNP | Ratio |
| None             | %   | %       | 0.4   | %   | %       | 0.2   |
| KCl              | 100 | 37      | 1.9   | 100 | 22      | 1.8   |
| NaCl             | 152 | 116     | 37    | 127 | 108     | 1.6   |
| LiCl             | 85  | 27      | 1.6   | 21  | 0.3     |       |
| MnCl₂            | 90  | 40      | 0.3   |      | 0.2     | 0.3   |

**Table III**

| Addition (5 mM) | Hydrolysis of Z-Gly-Gly-Leu-Amc | Hydrolysis of 14C-Casein |
|-----------------|---------------------------------|-------------------------|
| None            | %                               | %                       |
| EDTA            | 0.2                             | 0.3                     |
| MgCl₂           | 37                              | 15                      |
| MnCl₂           | 30                              | 15                      |
| CaCl₂           | 1.5                             | 1.5                     |

It is also noteworthy that an active form of HslVU can be induced without any nucleotide present, although nucleotide binding appears to be required for both HslU self-assembly and HslVU complex formation (16). The limited amount of the activity and of complex formation without a nucleotide present were apparently overlooked in earlier studies. While the absence of a divalent cation abolished all activity, Mg²⁺ prevented nucleotide binding to the enzyme. These findings indicate that Mg²⁺, like K⁺, stimulate the hydrolysis of Z-Gly-Gly-Leu-Amc and 14C-labeled casein in the presence of AMP-PNP but not ATP. Reaction mixtures contained 1 mM ATP or AMP-PNP and 50 mM salt as indicated. For peptidase assays, 1.5 μg/ml HslV and 4.3 μg/ml HslU were used; activity with ATP was assayed at 10 min and with AMP-PNP for varying times up to 40 min. For caseinase assays, 15 μg/ml HslV and 43 μg/ml HslU were incubated with 70 μg/ml 14C-labeled β-casein for 1 h. The activity obtained with ATP and no salt addition in each assay was expressed as 100%. The ratio of activity with AMP-PNP to that with ATP was then calculated.

It is also noteworthy that an active form of HslVU can be expressed as 100%. The ratio of activity with AMP-PNP to that with ATP was then calculated.

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