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ATP Binding, but Not Its Hydrolysis, Is Required for Assembly and Proteolytic Activity of the HslVU Protease in *Escherichia coli*

Soon Ji Yoo, Jae Hong Seol, Ihn Sik Seong, Man-Sik Kang, and Chin Ha Chung[1]
Department of Molecular Biology and Research Center for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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HslVU is an ATP-dependent protease consisting of two multimeric components: the HslU ATPase and the HslV peptidase. To gain an insight into the role of ATP hydrolysis in protein breakdown, we determined the insulin B-chain-degrading activity and assembly of HslVU in the presence of ATP and its nonhydrolyzable analogs. While β,γ-methylene-ATP could not support the proteolytic activity, β,γ-imido-ATP supported it to an extent less than 10% of that seen with ATP. Surprisingly, however, HslVU degraded insulin B-chain even more rapidly in the presence of ATPγS than with ATP. Furthermore, the ability of ATP and its analogs in supporting the proteolytic activity was closely correlated with their ability in supporting the oligomerization of HslU and the formation of the HslVU complex. However, ADP, which is capable of supporting the HslU oligomerization, could not support the HslVU complex formation or the proteolytic activity, suggesting that the conformation of the ADP-bound HslU oligomer is different from that of ATP-bound form. Thus, it appears that ATP-binding, but not its hydrolysis, is essential for assembly and proteolytic activity of HslVU.

Escherichia coli contains a number of ATP-dependent proteases, including protease La (Lon), which is a heat-shock protein composed of 4 identical 87-kDa subunits (1-3). This enzyme also is an ATPase that can be activated by degradable protein substrates but not by nondegradative polypeptides (4). Protease Ti (ClpAP), consists of two different multimeric components, both of which are required for ATP-dependent proteolysis (1-3,5,6). ClpA, which is composed of 84-kDa subunits, contains ATP-hydrolyzing sites, while ClpP, which is composed of 21-kDa subunits, is a serine protease. Like protease La, only degradable protein substrates activate the ATPase activity of ClpA alone or the reconstituted ClpAP complex (7). Moreover, the ability of the proteins to stimulate the ATPase activity is proportional to their susceptibility to the proteases. Therefore, it has been suggested that the hydrolysis of proteins and ATP by both proteases La and Ti occurs in a tightly linked fashion.

The heat shock protein HslVU in *E. coli* is a new type of ATP-dependent protease, consisting of a 19-kDa HslV and a 50-kDa HslU (8-11). HslU itself has ATPase activity, while HslV by itself is a weak peptidase that slowly degrades certain hydrophobic peptides, such as *N*-carbobenzoxy (Cbz)-Gly-Gly-Leu-7-amido-4-methylcoumarin (AMC). HslV alone also slowly degrades some polypeptides, including insulin B-chain and casein (12). In the reconstituted enzyme, HslU markedly stimulates the proteolytic activity of HslV (10- to 50-fold), while HslV increases the rate of ATP hydrolysis by HslU several-fold (9-11).

Similar to proteases La and Ti, the ATPase activity of HslU is stimulated by degradable polypeptide substrates but not by nondegradative proteins (12). Furthermore, ADP and *N*-ethylmaleimide, which inhibit ATP hydrolysis by HslU, prevent the ATP-dependent degradation of the polypeptide substrates, such as insulin B-chain, as well as of Cbz-Gly-Gly-Leu-AMC. Therefore, it has been suggested that the hydrolysis of proteins and ATP by HslVU also occurs in a coupled process. However, it has recently been revealed that β,γ-imido-ATP, a nonhydrolyzable ATP analog, can support the proteolytic activity of HslVU even better than ATP, although only in the presence of high concentrations of certain salts (*e.g.*, 0.2 M KCl), suggesting that proteolysis can be uncoupled from ATP cleavage (13).

In an attempt to clarify further the role of ATP hydrolysis in protein breakdown, the present study re-examined the effects of various nonhydrolyzable ATP analogs on the oligomerization of HslU, the formation of

[1] Corresponding author. Fax: (82) 2-872-1993.

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the HslVU complex, and their ability to support the proteolytic activity of the reconstituted enzyme.

MATERIALS AND METHODS

Assays. Peptide hydrolysis was assayed as described previously using Cbz-Gly-Gly-Leu-AMC as the substrate (9,10). Protein breakdown was assayed as described using ^{125}I-labeled insulin B-chain (12). ^{125}I-Labeled insulin B-chain was prepared using Iodo-Beads (Pierce) (14). Proteins were assayed by their absorbance at 280 nm or by the method of Bradford (15) using BSA as a standard.

Cross-linking analysis. HslV and HslU were purified as described previously (10). The purified HslU (4 μg), HslV (4 μg), or both were incubated in 0.1 M HEPES buffer (pH 7.5) containing 10 mM $MgCl_2$, 1 mM EDTA, and 0.4% (v/v) glutaraldehyde in the presence and absence of various adenine nucleotides in a total volume of 0.1 ml. After incubation for 20 min at 37°C, the samples were mixed with 30 μl of 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% 2-mercaptoethanol. They were then subjected to polyacrylamide gel electrophoresis on 4-8% (w/v) gradient slab gels containing SDS and 2-mercaptoethanol (16). Proteins in the gels were then visualized by silver staining.

RESULTS AND DISCUSSION

Effects of ATP and Its Analogs on the Hydrolysis of Insulin B-chain

We have previously shown that ADP and some nonhydrolyzable ATP analogs, including β,γ-methylene-ATP (AMP-PCP) and β,γ-imido-ATP (AMP-PNP), cannot support the hydrolysis of Cbz-Gly-Gly-Leu-AMC by the HslVU protease, and therefore suggested that ATP cleavage is required for the peptide hydrolysis (9,10). In an attempt to clarify further the role of ATP hydrolysis in protein breakdown, we again examined the ability of various ATP analogs at 1 mM in supporting the proteolytic activity of the enzyme using ^{125}I-labeled insulin B-chain as a substrate. In accord with the earlier finding on the cleavage of Cbz-Gly-Gly-Leu-AMC (8,9), AMP-PCP was unable to support the hydrolysis of insulin B-chain by the HslVU protease (Fig. 1). In addition, AMP-PNP supported the activity less than 10% of that seen with ATP. However, the HslVU protease degraded insulin B-chain even more rapidly in the presence of ATPγS than with ATP. Similar data were obtained when Cbz-Gly-Gly-Leu-AMC or β-casein was used as a substrate (data not shown). These results suggest that ATP hydrolysis is not essential for peptide bond cleavage.

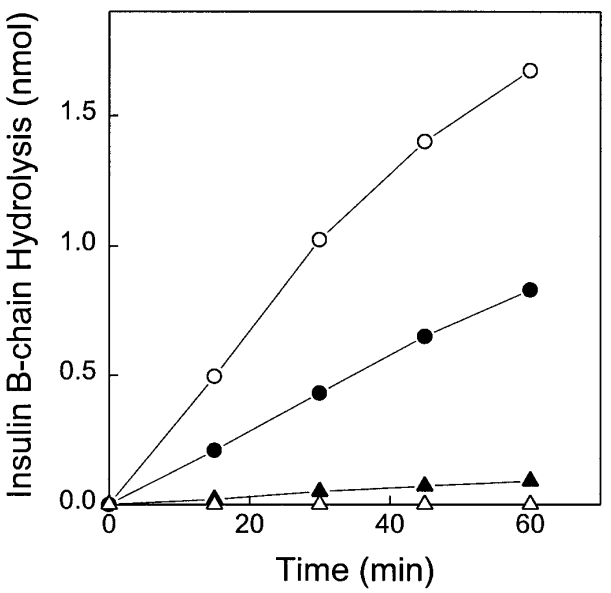

FIG. 1. Time-course for the hydrolysis of insulin B-chain by the HslVU protease in the presence of ATP and its analogs. Reaction mixtures containing 10 μg of ^{125}I-labeled insulin B-chain (2-3 $\times$ 10^3 cpm/μg), 1 μg of HslV, and 4 μg of HslU were incubated at 37 °C for various period in the presence of 1 mM ATP (●), ATPγS (○), AMP-PNP (▲), or AMP-PCP (△). The radioactivity released into acid-soluble products were then determined as described (12).

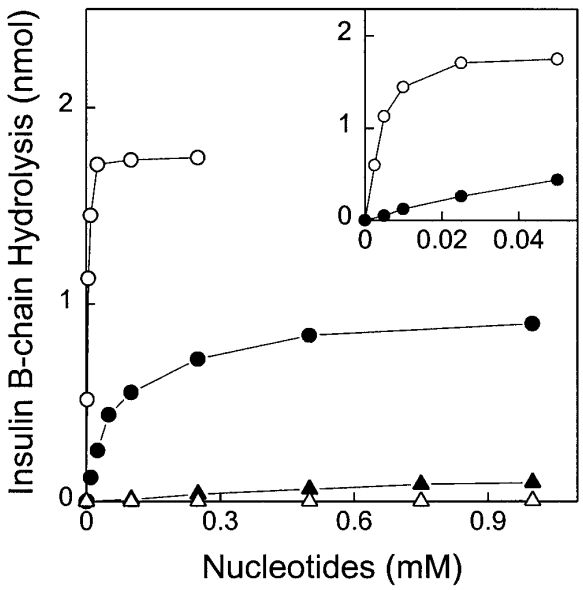

FIG. 2. Effects of increasing concentrations of ATP and its analogs on the hydrolysis of insulin B-chain. Assays were performed as in Fig. 1 but in the presence of increasing concentrations of ATP (●), ATPγS (○), AMP-PNP (▲), or AMP-PCP (△). The inset indicates the enzyme activity at lower concentrations of ATP (●) and ATPγS (○).

Since the chemical nature brought into the ATP molecule for preventing its cleavage is different among the ATP analogs, we suspected that the affinity of the analogs to the HslVU protease may differ from each other and hence their ability to support the proteolytic activity of the enzyme may also be different. Therefore, we examined the effects of the increasing concentrations of the nonhydrolyzable ATP analogs on degradation of insulin B-chain. Fig. 2 shows that the proteolytic activity of HslVU becomes maximal at much lower concentrations of ATPγS than ATP. Upon double reciprocal plots of the data, the concentrations of ATPγS and ATP that are required to achieve a half maximal activity

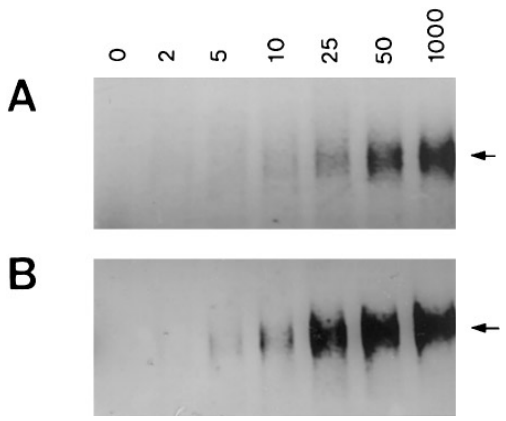

FIG. 3. Cross-linking analysis for the oligomerization of HslU in the presence of increasing concentrations of ATP and its analogs. HslU (4 μg) was incubated for 20 min at 37 °C with 0.4% (v/v) glutaraldehyde in the presence of increasing concentrations of ATP (A) and ATPγS (B). The samples were then electrophoresed as described under Materials and Methods. Proteins in the gels were visualized by silver staining. The numerals on the top of the gel indicate the concentrations of adenine nucleotides (μM).

were estimated to be approximately 3 and 70 μM, respectively. Thus, it appears that ATPγS shows much higher affinity to the HslVU protease than ATP and this elevated affinity due to the γ-thiophosphate substitution in ATP molecule may be responsible for the increased proteolytic activity of the enzyme.

Effects of ATP and Its Analogs on the Oligomerization of HslU

The purified HslU alone has been shown to exist as monomer and/or dimer in the absence of ATP but as an oligomer containing 6-7 subunits in its presence (17-19). In order to determine whether HslU can form an oligomeric complex more efficiently in the presence of ATPγS than with ATP, cross-linking analysis was performed using glutaraldehyde in the presence of increasing amounts of the adenine nucleotides. Fig. 3 shows that HslU forms an oligomer in the presence of much lower concentrations of ATPγS than with ATP. For example, at 25 μM, the amount of the oligomerized HslU is much higher in the presence of ATPγS than with ATP. On the other hand, much less amount of HslU formed an oligomer even at 1 mM AMP-PNP, compared to that with either of the nucleotides (data not shown and see below). These results suggest that the affinity of the adenine nucleotides is closely correlated with their ability in supporting the oligomerization of HslU. These results also suggest that oligomerization of HslU is a requisite step for its interaction with HslV and hence for the proteolytic activity of the HslVU protease complex.

Formation of the HslVU Complex in the Presence of ATP and Its Analogs

In order to determine whether the oligomerization of HslU is required for the formation of the HslVU complex, HslU, HslV, or both were incubated in the presence and absence of ATP or its analogs, followed by cross-linking analysis. In the absence of any adenine nucleotide, HslU could not form an oligomer, whether or not HslV was present (Fig. 4A, lanes a and c). Under the same condition, HslV formed a 210-kDa oligomer (*i.e.*, a dodecamer) in addition to other cross-linked intermediates, such as 120-kDa hexamer, whether or not HslU was present (lanes b and c). In the presence of 1 mM ATP or its analogs, however, incubation of HslU with HslV resulted in a formation of a new, high molecular mass protein band that should represent the HslVU complex (Fig. 4C, indicated by an arrow). Furthermore, the amounts of the complex generated in the presence of ATP and its analogs were correlated with their varied ability in supporting the oligomerization of HslU (Figs. 4B and 4C, lanes d, f, and g) as well as the proteolytic activity of the HslVU protease (see Fig. 1). These results strongly suggest that the oligomerization of HslU is essential for the HslVU complex formation.

However, despite the finding that ADP supports the oligomerization of HslU itself (Fig. 4B, lane e), the oligomeric HslU could not form a complex with HslV in the presence of the adenine dinucleotide (Fig. 4C, lane e). One possible explanation for the differential effects of the adenine nucleotides on the HslVU complex formation is

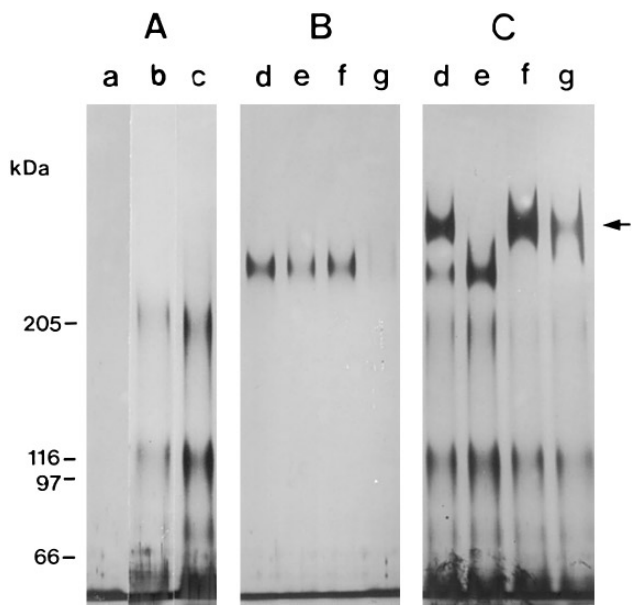

FIG. 4. Cross-linking analysis for the formation of the HslVU complex in the presence of ATP and its analogs. Aliquots (4 μg each) of HslU (lane a), HslV (lane b), or both (lane c) were incubated with 0.4% glutaraldehyde for 20 min at 37 °C in the absence any nucleotide (A). HslU alone (B) or with HslV (C) was incubated as above in the presence of 1 mM ATP (lanes d), ADP (lanes e), ATPγS (lanes f), and AMP-PNP (lanes g). After incubation, the samples were electrophoresed under denaturing conditions followed by silver-staining. Note that HslU was stained much poorly than HslV by silver nitrate for an unknown reason(s). The arrow indicates the HslVU complex.

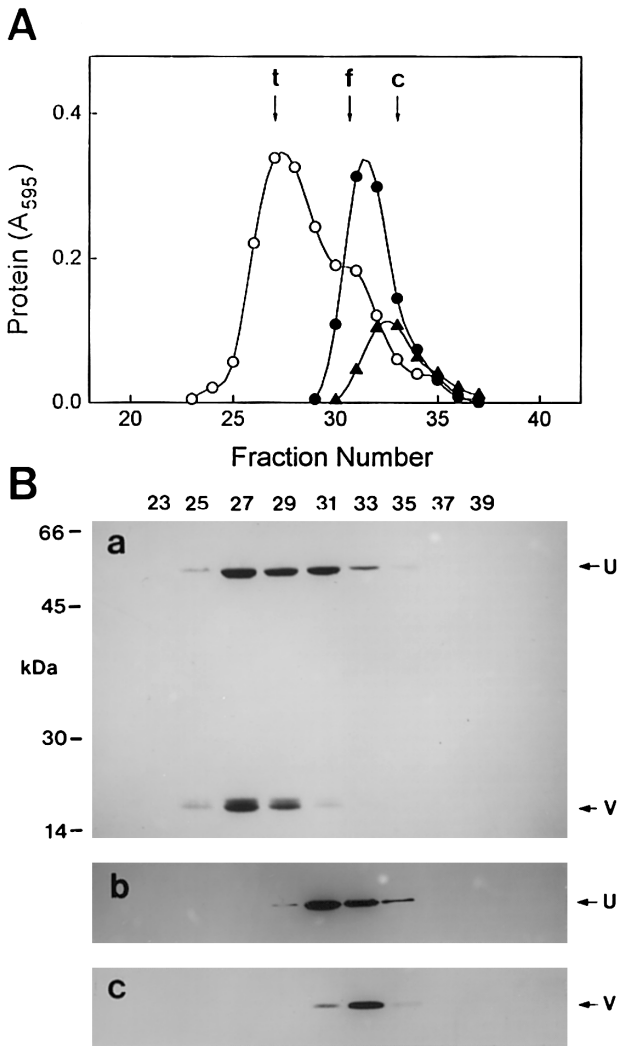

FIG. 5. Gel filtration analysis for the assembly of the HslVU protease complex in the presence of ATPγS. (A) HslU (150 μg) (●), HslV (50 μg) (▲), or both (○) were incubated with 0.1 mM ATPγS for 20 min at 37 °C. After incubation, each sample was chromatographed on a Superose-6 column (1 × 30 cm) that had been equilibrated with 0.1 M HEPES buffer (pH 7.5) containing 0.1 mM ATPγS, 10 mM $MgCl_2$, and 1 mM EDTA. The size markers used were: t, thyroglobulin (669 kDa); f, ferritin (440 kDa); c, catalase (232 kDa). (B) Aliquots of the column fractions under the protein peaks were subjected to polyacrylamide gel electrophoresis on 13% slab gels containing SDS and 2-mercaptoethanol. Proteins in the gels were then stained with Coomassie blue R-250.

that the conformation of the ADP-bound HslU oligomer is different from that of the ATP- or ATPγS-bound form and therefore cannot interact with HslV.

In order to confirm that HslV and HslU can indeed form a complex in the presence of ATPγS, we incubated HslV, HslU, or both in the presence of the ATP analog (0.1 mM) but without glutaraldehyde. After incubation, the samples were subjected to gel filtration on a Superose 6 column, followed by gel electrophoresis of the column fractions under denaturing conditions. HslU alone ran as an oligomer with a size of 300-350 kDa (Fig. 5A, closed circle), as was previously found upon incubation with ATP (10,12,17). In addition, HslV alone ran as a 250-kDa protein whether or not ATPγS was present (Fig. 5A, triangle and data not shown). When HslU and HslV were incubated together in the presence of ATPγS, there appeared a new protein peak with a size of about 600 kDa (Fig. 5A, open circle), which corresponds to the sum of the molecular masses of HslV and HslU. Furthermore, both HslV and HslU were found in the new peak fractions upon the gel electrophoretic analysis (Fig. 5B, panel a). However, no such protein peak appeared in the fractions corresponding to 600 kDa, when HslV and HslU were incubated in the presence of ADP (data not shown). Thus, it seems clear that HslV and HslU can form a stable complex in the presence of ATPγS.

Effect of ADP on the Assembly of the HslVU Protease Complex

We have recently shown that ADP inhibits not only ATP hydrolysis but also degradation of Cbz-Gly-Gly-Leu-AMC and insulin B-chain by the HslVU protease (12). Furthermore, the inhibitory effects on ATP cleavage and proteolysis were found to increase in parallel with the increase in ADP concentration. Therefore, we examined whether the inhibitory effect of ADP is due

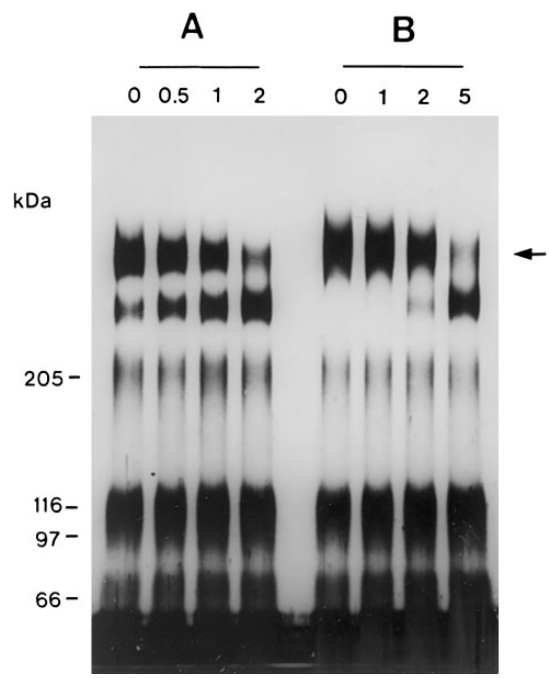

FIG. 6. Effect of ADP on the formation of the HslVU complex in the presence of ATP or ATPγS. HslV (4 μg) and HslU (4 μg) were incubated with 1 mM ATP (A) or ATPγS (B) and 0.4% glutaraldehyde in the presence of increasing concentrations of ADP for 20 min at 37 °C. The numerals on the top of the gel indicate the ADP concentrations (mM). The arrow indicates the HslVU complex.

to its competition with ATP or the analogs in binding to HslU and hence in the formation of the HslVU complex. To test this possibility, HslV and HslU were incubated with ATP or ATPγS in the presence of increasing concentrations of ADP, followed by cross-linking analysis. As shown in Fig. 6, ADP reduced the ability of ATP and ATPγS in supporting the HslVU complex formation in a concentration-dependent fashion. However, the concentration of ADP that is required for preventing the formation of the ATPγS-bound complex was much higher than that for inhibiting the ATP-bound complex formation, further indicating that ATPγS has a higher affinity to HslU than ATP. Thus, it appears likely that the inhibitory effect of ADP on the proteolytic activity of the HslVU protease is mediated by prevention of the enzyme complex formation. In this respect, ATP-binding and its subsequent hydrolysis by the HslVU protease seem to play a role in rapid association-and-dissociation of the enzyme complex for recycling of the enzyme in the process of degradation of protein substrates, and/or for prevention of excessive breakdown of cell proteins. Alternatively, ATP hydrolysis by HslU may be required for its possible function as a chaperone, like the ClpA (20) and ClpX ATPases (21) in *E. coli*.

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