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Role of the GYVG Pore Motif of HslU ATPase in Protein Unfolding and Translocation for Degradation by HslV Peptidase*

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Eunyong Park‡§, Young Min Rho‡§, Ohn-jo Koh‡, Sung Won Ahn‡, Ihn Sik Seong‡, Ji-Joon Song¶, Oksun Bang‡, Jae Hong Seol‡, Jimin Wang‖, Soo Hyun Eom¶, and Chin Ha Chung‡

From the ‡NRL of Protein Biochemistry, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea, the ‖Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, and the ¶Department of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-751, Korea

HslVU is an ATP-dependent protease consisting of HslU ATPase and HslV peptidase. In an HslVU complex, the central pores of HslU hexamer and HslV dodecamer are aligned and the proteolytic active sites are sequestered in the inner chamber of HslV. Thus, the degradation of natively folded proteins requires unfolding and translocation processes for their access into the proteolytic chamber of HslV. A highly conserved GYVG^{93} sequence constitutes the central pore of HslU ATPase. To determine the role of the pore motif on protein unfolding and translocation, we generated various mutations in the motif and examined their effects on the ability of HslU in supporting the proteolytic activity of HslV against three different substrates: SulA as a natively folded protein, casein as an unfolded polypeptide, and a small peptide. Flexibility provided by Gly residues and aromatic ring structures of the 91st amino acid were essential for degradation of SulA. The same structural features of the GYVG motif were highly preferred, although not essential, for degradation of casein. In contrast, none of the features were required for peptide hydrolysis. Mutations in the GYVG motif of HslU also showed marked influence on its ATPase activity, affinity to ADP, and interaction with HslV. These results suggest that the GYVG motif of HslU plays important roles in unfolding of natively folded proteins as well as in translocation of unfolded proteins for degradation by HslV. These results also implicate a role of the pore motif in ATP cleavage and in the assembly of HslVU complex.

A number of ATP-dependent proteases have been identified and studied structurally and mechanically, including the 26 S proteasome in eukaryote and Lon, FtsH, ClpAP, ClpXP, and HslVU in prokaryote (1–3). These proteases play essential roles in controlling the levels of key regulatory proteins and in the elimination of abnormal polypeptides (3–5). Of these, HslVU consisting of HslV peptidase and HslU ATPase resembles the 26 S proteasome in their sequence and overall architecture (6–8). HslV consists of two doughnut-shaped hexameric rings, within which the proteolytic active sites are sequestered with access to this chamber restricted to small axial pores (9–11).

HslU ATPase belongs to the ATPase-associated cellular activity plus superfamily (AAA+) (12, 13). HslU stimulates the proteolytic activity of HslV one to two orders of magnitude, regardless of whether the substrate is a short peptide or protein that must be unfolded (14). As HslU markedly stimulates the proteolytic activity of HslV, in turn HslV increases the rate of ATP hydrolysis by HslU severalfold (6). In the absence of HslV, HslU also serves as a molecular chaperone in preventing the aggregation of SulA, a cell division inhibitor protein that has an intrinsic tendency of aggregation *in vivo* as well as *in vitro* (15).

HslU forms a hexameric ring that constitutes a small central pore (Fig. 1*A*). The central translocation pore of HslU consists of a primary and a secondary motif (16). The primary motif contains a conserved GYVG sequence that is located next to Walker box A, and the secondary motif is located in the sequence next to Walker box B (Figs. 1*C* and 2*A*). In an HslVU complex, the central pores of HslU and HslV are aligned next to each other but their diameters are so small that only a single polypeptide can be threaded through. Therefore, protein substrates with native structure must be unfolded to an extended conformation and translocated into the inner chamber of HslV for proteolysis.

The crystal structures of HslU hexamer have revealed that a large nucleotide-dependent conformational change is transmitted from the nucleotide binding pockets to the rest of the structure, including the pore region containing the GYVG motif and the C-terminal tail region (10, 11, 17, 18). Depending on the type and content of bound adenine nucleotides, HslU conformation interconverts between "open" and "closed" states according to the pore geometry. This interconversion coincides with an up-down movement of the central pore region of HslU relative to the aligned pore of the bound HslV (17). In particular, Tyr^{91} in the GYVG pore motif shows a marked movement from inside HslU toward HslV upon the conversion of HslU conformation from the open to closed state (Fig. 1*B*). Therefore, we have previously proposed that the inter-conversion of HslU conformation driven by ATP hydrolysis may provide a direct force for unfolding and translocation of protein substrates.

In an attempt to determine the role of the GYVG pore motif of HslU in protein unfolding and translocation, we generated a number of mutations in the motif sequence and examined their effects on the ability of HslU in supporting the HslV-mediated degradation of three different types of the substrates: SulA as a natively folded protein that requires both unfolding and translocation, casein as an unfolded protein requiring only translocation, and *N*-carbobenzyloxy-Gly-Gly-Leu-7-amido-4-

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** To whom correspondence should be addressed. Tel.: 82-2-880-6693; Fax: 82-2-871-9193; E-mail: chchung@snu.ac.kr.

methylcoumarin (Z[1]-Gly-Gly-Leu-AMC) as a peptide substrate that requires neither unfolding nor translocation. We also examined the effects of the mutations on ATP hydrolysis by HslU in the absence and presence of HslV and on the formation of HslVU complex.

EXPERIMENTAL PROCEDURES

Materials—All reagents for PCR, including *Taq* polymerase and restriction endonucleases, were purchased from Takara. Peptide substrates were obtained from Bachem Feinchmikalien AG. Reagents required for ATPase assay were purchased from Molecular Probes. All other reagents were purchased from Sigma, unless otherwise indicated.

Mutagenesis and Protein Purification—The pGEM-T/HslVU vector was constructed as described previously (6). Site-directed mutagenesis was carried out using QuikChange site-directed mutagenesis kit (Stratagene) and pGEM-T/HslVU as a template. The PCR reactions were carried out using mutagenic primers. The resulting plasmids were transformed into MC1000H. Substitutions of the nucleotides by mutagenesis were verified by DNA sequencing. *Escherichia coli* strains MC1000H (*hslVU::kan*) and MC1000LH (*lon::tet, hslVU::kan*) were constructed as described (19). HslU, its mutant forms, and HslV were purified as described (6, 20). The pMAL-p2-SulA vector encoding MBP-SulA was obtained from Dr. Higashitani (21). MBP-SulA was purified as described (19).

Assays—Peptide hydrolysis was assayed using Z-Gly-Gly-Leu-AMC as a substrate (6). Reaction mixtures (0.1 ml) contained 0.1 mM peptide, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 1 mM EDTA, and appropriate amounts of the purified HslV and HslU or its mutant forms in 0.1 M Tris-HCl buffer (pH 8). The release of AMC from the peptide was continuously measured by incubation of the mixtures at 37 °C ($\lambda_{excitation}$ = 355 nm; $\lambda_{emission}$ = 460 nm) using a fluorometer (Fluostar Optima, BMG LABTECH). The rate of peptide hydrolysis was determined by averaging initial slopes of four independent measurements.

ATP hydrolysis was assayed using EnzChek® phosphate assay kit (Molecular Probes) with some modification (22). Reaction buffers contained 0.1 M Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, and 1 mM ATP. To remove contaminated inorganic phosphates, the buffers were treated with 0.2 mM 2-amino-6-mercapto-7-methylpurine riboside and 1 unit of purine nucleoside phosphorylase followed by incubation at 37 °C for 10 min. The samples were added with HslV (22 nM) and HslU or its mutant forms (67 nM), and the release of free phosphates from ATP was then monitored by continuous measurement of the absorbance at 360 nm at 37 °C using a spectrophotometer (Ultrospec 2000). The rate of ATP hydrolysis was determined by averaging initial slopes of three independent measurements.

The degradation of MBP-SulA and α-casein was assayed by incubating reaction mixtures (0.1 ml) for appropriate periods at 37 °C (15). The reaction mixtures contained 0.97 μM MBP-SulA or 4.3 μM α-casein, 44 nM HslV, and 0.13 μM HslU or its mutant forms in 0.1 M Tris-HCl buffer (pH 8), 10 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 1 mM EDTA, 30 mM creatine phosphate, and 0.52 μM creatine phosphokinase. After incubation, the reaction was stopped by adding 30 μl of 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% (v/v) 2-mercaptoethanol. The samples were then subjected to SDS-PAGE on 12% slab gels (23), followed by staining with Coomassie Blue R-250. Proteins were quantified by the method of Bradford (24) using bovine serum albumin as a standard.

Cross-linking Analysis—Cross-linking analysis was performed as described (25). Reaction mixtures containing 0.13 μM HslU or its mutant forms in 0.1 M HEPES buffer (pH 8), 10 mM $MgCl_2$, 0.2 M NaCl, 1 mM DTT, and 1 mM EDTA were incubated in the absence or presence of 0.18 μM HslV, ATPγS, or both for 5 min at 37 °C. After incubation, the mixtures were treated with glutaraldehyde (0.4% in a total volume of 0.1 ml) and further incubated for 20 min at 37 °C. They were then mixed with 30 μl of 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% 2-mercaptoethanol and subjected to SDS-PAGE on 4–8% gradient slab gels. Proteins in the gels were visualized by silver staining.

Size Exclusion Chromatography—Gel filtration was performed using Waters 626 HPLC equipped with a KW-804 GFC column (Shodex) at room temperature. Aliquots (0.1 ml) of HslU or its mutant forms (1.67 μM) were injected to the column that had been equilibrated with 30 mM Tris-HCl (pH 7.6), 0.3 M NaCl, 1 mM EDTA, and 1 mM DTT. When necessary, 1 mM ATP was also included in the buffer. Fractions (82 μl each) were collected at a flow rate of 0.5 ml/min and then subjected to SDS-PAGE.

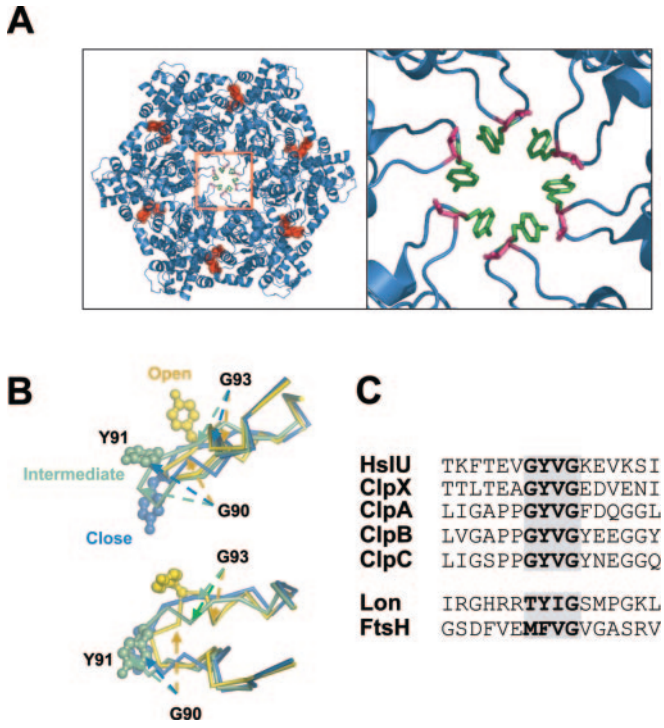

FIG. 1. **Structure of HslU.** *A*, x-ray crystal structure of an HslU hexamer (Protein Data Bank code 1G4A) was viewed from the distal side to HslV in the *left panel*. Bound ADP molecules are shown as *red*. The GYVG pore motif was enlarged in the *right panel*. *Green* and *pink* indicate Tyr^{91} and Val^{92}, respectively. The figure was drawn by using PyMOL (51). *B*, Tyr^{91} in the GYVG motif shows a marked movement from the inside of HslU toward HslV upon conversion of HslU conformation from the open to closed states. The *upper* and *lower panels* show the GYVG motif viewed from two different angles. *C*, the amino acid sequences of the primary pore motif in various ATPases were shown. The conserved GYVG sequences were *shaded*.

RESULTS

Generation of Mutations in GYVG Pore Motif—To determine the importance of the GYVG motif of HslU in protein unfolding and translocation, site-directed mutagenesis was performed to replace each residue in the pore motif as below. Since a large motion of Tyr^{91} in the GYVG pore motif is apparent during nucleotide-dependent interconversion of HslU conformation between the open and closed states (17), we speculated that Gly^{90} and/or Gly^{93} might provide the flexibility for the motion. In addition, both Gly residues are strictly conserved in all of the ATPases of two component ATP-dependent proteases, whereas only Gly^{93} is conserved in Lon and FtsH, the ATP-dependent proteases that have the ATPase and protease domains within a single polypeptide (Fig. 1*C*). Therefore, we replaced both Gly residues by Pro with an expectation that the rigid residues might obstruct the movement of the GYVG motif (Fig. 2*A*). We also replaced Gly^{93} by Ala. Sequence comparison also revealed that FtsH has a Phe residue in place of Tyr at the second position of the GYVG motif, while Lon contains Ile in place of Val at its third position (Fig. 1*C*). Therefore, we replaced Tyr^{91} and Val^{92} by Phe and Ile (Fig. 2*A*). The Tyr and Val residues were also replaced by Cys, Ala, and Ser, which have relatively small side chains. All of the HslU mutant proteins were then purified to apparent homogeneity (Fig. 2*B*).

Effects of GYVG Mutations on Degradation of SulA, a Natively Folded Protein—SulA is a cell division inhibitor encoded by the SOS-inducible *sfiA* gene (26, 27). HslVU as well as Lon have previously been shown to degrade the inhibitor protein

[1] The abbreviations used are: Z, *N*-carbobenzyloxy; AMC, 7-amido-4-methylcoumarin; DTT, dithiothreitol; MBP, maltose-binding protein; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

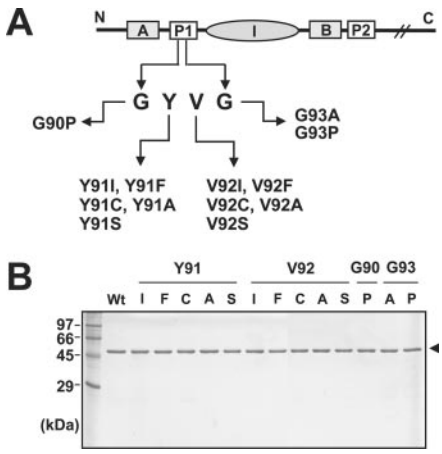

FIG. 2. **Mutagenesis of the GYVG motif in HslU and purification of the mutant proteins.** *A*, the primary sequence of HslU was shown as a schematic diagram. The GYVG sequence in P1 motif was subjected to mutagenesis as indicated. The *boxed A* and *B* indicate Walker boxes A and B, respectively. The *open boxed P1* and *P2* show the pore motif-1 and -2, respectively. The *oval I* indicates the intermediate domain. *B*, wild-type HslU and its mutant forms were purified and subjected to SDS-PAGE as described under "Experimental Procedures." The *arrowhead* indicates the purified HslU proteins. Wild-type HslU is indicated as *Wt*, and its mutant forms as the *single capital letters* at the respective sites.

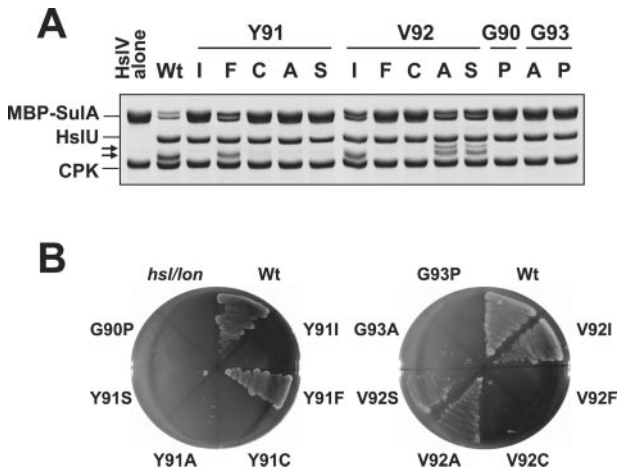

FIG. 3. **Effects of the mutations in the GYVG motif on the ability of HslU in supporting the HslV-mediated degradation of SulA and on cell survival.** *A*, wild-type HslU (*Wt*) and its mutant proteins (0.13 μM) were incubated with 44 nM HslV, 0.97 μM MBP-SulA, 2 mM ATP, and ATP regeneration system at 37 °C for 1.5 h. After incubation, the samples were subjected to SDS-PAGE as described under "Experimental Procedures." The *arrows* indicate the degradation products of SulA. *CPK* denotes creatine phosphokinase. *B*, MC1000LH (*lon::tet, hslVU::kan*) cells were transformed with the cDNAs for HslV and wild-type HslU (*Wt*) or its mutant forms. The cells were then grown overnight on agar plates containing nitrofurantoin (2 μg/ml).

(19, 28, 29). To determine the effects of the mutations in the GYVG motif on the degradation of proteins with natively folded structures, the HslU mutant proteins were incubated with SulA and HslV. Among the Tyr^{91} and Val^{92} mutant proteins, Y91F, V92I, V92A, and V92S could support the SulA degradation by HslV although to less extents than wild-type HslU (Fig. 3*A*). The inability of the Tyr^{91} mutant proteins, except Y91F, in supporting the HslV-mediated SulA degradation suggests that aromatic ring structures of the 91st residue are essential for the degradation of natively folded proteins, like SulA. On the other hand, the finding that V92A and V92S are capable of supporting the proteolysis suggests the inessentiality of hydrophobic Val^{92}, although it remains unclear why V92F and V92C cannot support it. None of the Gly^{90} or Gly^{93} mutant proteins could support the proteolysis. Thus, it appeared that the flexibility of the 90th and 93rd Gly residue is also required for degradation of native proteins.

When *E. coli* cells are under SOS conditions (*e.g.* UV irradiation or treatment with UV mimicking agents, such as nitrofurantoin), SulA accumulates and blocks the cell growth by inhibiting cell division (26, 29). To determine whether the expression of the HslU mutant proteins with HslV could rescue from the cell growth inhibition under SOS conditions, *hsl/lon* cells were transformed with the cDNAs for the HslU mutant proteins and HslV and grown on agar plates containing nitrofurantoin. As shown in Fig. 3*B*, cells expressing Y91F, V92I, V92A, or V92S, but not the others, could survive under the SOS condition. These results suggest that Y91F, V92I, V92A, and V92S are capable of supporting the HslV-mediated SulA degradation *in vivo* as well as *in vitro*.

Effects of GYVG Mutations on Degradation of Casein, an Unfolded Protein—Unlike SulA that requires both unfolding and translocation for its degradation, unfolded proteins like casein would need only the translocation step for their access into the inner proteolytic chamber of HslV. Casein is known as a natively unfolded protein (30). Therefore, we examined the ability of the HslU mutant proteins in supporting the degradation of unfolded proteins using casein as a substrate. All of the Tyr^{91} and Val^{92} mutant proteins were capable of supporting the casein degradation by HslV although to varied and lesser extents than wild-type HslU (Fig. 4). Significantly, the preference of amino acids at the 91st and 92nd positions in supporting the casein hydrolysis was similar to that in supporting the SulA degradation. Y91F, V92I, V92A, and V92S, which could support the SulA degradation, were also capable of supporting the casein hydrolysis much better than the others that were unable to support the SulA degradation. These results suggest that unfolding and translocation of protein substrates may occur as a closely coupled process, although the translocation process is less stringent than the unfolding step in the requirement of specific amino acids at the 91st and 92nd positions of the GYVG pore motif.

None of the Gly^{90} or Gly^{93} mutant proteins could support the HslV-mediated casein hydrolysis, suggesting that the flexibility provided by both Gly residues might be required for the translocation of unfolded polypeptides. However, the inability of G93A and G93P in supporting the hydrolysis of casein as well as of SulA appeared to be due to their inability to form an HslVU complex with HslV (see below).

Effects of GYVG Mutations on Hydrolysis of Z-Gly-Gly-Leu-AMC—To determine the effects of the mutations in the GYVG motif on the hydrolysis of small peptides, the HslU mutant proteins were incubated with Z-Gly-Gly-Leu-AMC and HslV. All of the Tyr^{91} and Val^{92} mutant proteins supported the HslV-mediated peptide hydrolysis although to varied extents (Fig. 5*A*). Interestingly, certain HslU mutant proteins (*e.g.* Y91A and Y91S), which supported the casein degradation less than 25% as well as wild-type HslU, were capable of supporting the peptide hydrolysis nearly twice better than HslU. However, it is unlikely that the preference of the amino acids for peptide hydrolysis is related with the translocation of peptide substrates, because small peptides would not require any facilitated translocation as long as the diameters of the central pores of HslU and HslV are sufficiently large enough for their passage. G90P could support the peptide hydrolysis about 60% as well as wild-type HslU, suggesting that the flexibility of the Gly residue is not essential for peptide hydrolysis. In contrast, neither G93A nor G93P was capable of supporting the peptidase activity of HslV. However, the inability of G93A and G93P

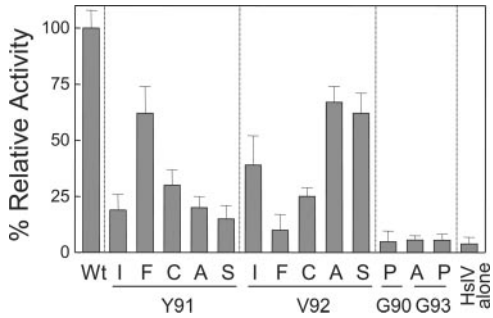

FIG. 4. **Effects of the mutations in the GYVG motif on the ability of HslU in supporting the HslV-mediated degradation of casein.** Wild-type HslU (*Wt*) and its mutant forms (0.13 μM) were incubated with 44 nM HslV, 4.3 μM α-casein, 2 mM ATP, and ATP regeneration system at 37 °C for 1 h. After incubation, the samples were subjected to SDS-PAGE and analyzed by densitometry. The data represent the averaged values of three independent measurements, and their standard deviations are shown as *error bars*.

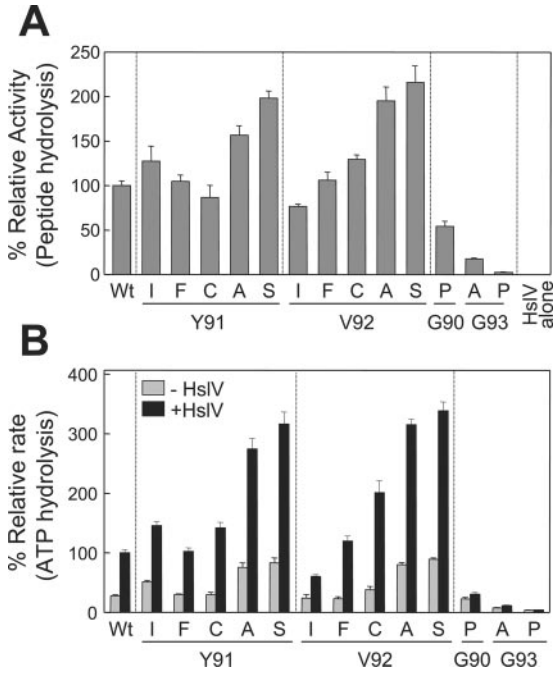

FIG. 5. **Effects of the mutations in the GYVG motif on the ability of HslU in supporting the HslV-mediated degradation of Z-Gly-Gly-Leu-AMC and on the rate of ATP hydrolysis.** *A*, wild-type HslU and its mutant proteins (13.5 nM) were incubated with 4.4 nM HslV, 0.1 mM Z-Gly-Gly-Leu-AMC, and 1 mM ATP at 37 °C. The release of AMC from the peptide was continuously measured using a fluorometer. The rates of peptide hydrolysis were expressed relative to those seen with wild-type HslU and HslV, which were expressed as 100%. *B*, wild-type HslU and its mutant proteins (67 nM) were incubated with 1 mM ATP in the absence (*gray bars*) or presence of 22 nM HslV (*black bars*) at 37 °C. The phosphate released was then assayed as described under "Experimental Procedures." All values are the averages of four independent measurements, and their standard deviations are shown as *error bars*.

in supporting the peptide hydrolysis appeared to be due to their inability to form HslVU complex with HslV (see below).

Effects of GYVG Mutations on ATP Hydrolysis—Since the GYVG motif is located next to Walker box A (*i.e.* P loop motif where ATP binds), we examined whether the mutations in the pore motif might influence the ATPase activity of HslU. The effects of the mutations on ATP cleavage were remarkably well correlated with their effects on the ability of HslU in supporting the hydrolysis of Z-Gly-Gly-Leu-AMC by HslV. The replacement of Tyr^{91} and Val^{92} by Ala or Ser, which caused about 2-fold increase in the ability of the HslV-mediated peptide

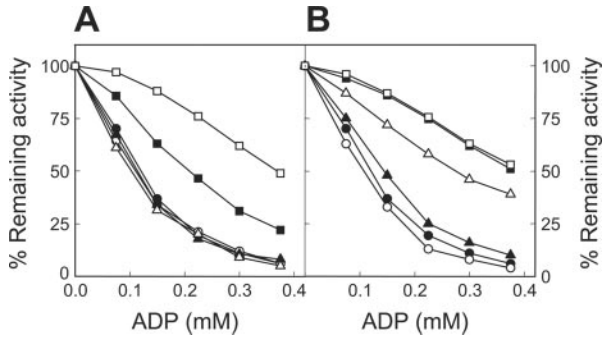

FIG. 6. **Effect of ADP on the ATPase activity of HslU and its mutant forms.** Wild-type HslU and its mutant proteins (67 nM) were incubated with 1 mM ATP and 22 nM HslV in the presence of increasing concentrations of ADP at 37 °C. The phosphate released was then determined. The ATP hydrolyzing activities were expressed relative to those seen with ATP only, which were expressed as 100%. *A* and *B* show the activities of the Tyr^{91} and Val^{92} mutant proteins, respectively, which were generated by the replacement of each of the amino acids by Ser (□), Ala (■), Cys (△), Phe (▲), and Ile (○). The activity of wild-type HslU (●) was also shown. Similar data were obtained in three independent experiments.

hydrolysis, increased both the basal and HslV-activated ATPase activities of HslU by about 3-fold (Fig. 5*B*). On the other hand, the replacement of Val^{92} by Ile and that of Gly^{90} by Pro resulted in a decrease in the ability of HslU to cleave ATP as well as in its ability to support the peptide hydrolysis although to different extents. Moreover, G93A and G93P, which were unable to support the peptide hydrolysis, showed little or no ATPase activity whether or not HslV was present. Thus, it appeared as if the ability of the HslU proteins in supporting the HslV-mediated peptide hydrolysis was tightly coupled with their ability to cleave ATP.

However, we have previously shown that ATP binding to HslU, but not its hydrolysis, is required for HslV-mediated peptide hydrolysis (31). In this respect, we suspected if the changes in the ability of HslU mutant proteins in supporting the peptide hydrolysis might be due to an alteration in the binding affinity of ATP to HslU. To test this possibility, ATP hydrolysis was assayed by incubating wild-type HslU and its mutant forms with increasing concentrations of ATP in the absence or presence of HslV. The K_m values of ATP estimated by double reciprocal analysis were not significantly changed by the mutations of Gly^{90}, Tyr^{91}, and Val^{92} (data not shown), *i.e.* the difference was within 30% of that for wild-type HslU, which was 280 μM. These results suggest that the mutations may not grossly alter the affinity of ATP to HslU.

ADP binds to HslU with a higher affinity than ATP and acts as a strong inhibitor of HslU ATPase (7). In an attempt to determine whether the mutations in the GYVG motif might alter the binding affinity of ADP to HslU, ATP hydrolysis was assayed by incubating HslU or its mutant forms with HslV in the presence of 1 mM ATP and increasing concentrations of ADP. The HslU mutant proteins that showed higher ATPase activity than wild-type HslU (*i.e.* Y91S, Y91A, V92S, V92A, and V92C) were much less sensitive to inhibition by ADP, compared with the other mutant proteins and wild-type HslU (Fig. 6). From these data, half-maximal inhibitory concentrations of ADP (IC_{50}) against the HslV-activated ATPase activity of these mutant proteins were estimated to be ~2–4-fold higher than those of the other mutant proteins and wild-type HslU. For example, IC_{50} for wild-type HslU and Y91S were about 0.12 mM and 0.38 mM, respectively. These results suggest that the replacement of Tyr^{91} or Val^{92} by amino acids having relatively small side chains causes a decrease in the affinity of ADP to HslU, which may in turn result in the increase in the ATPase

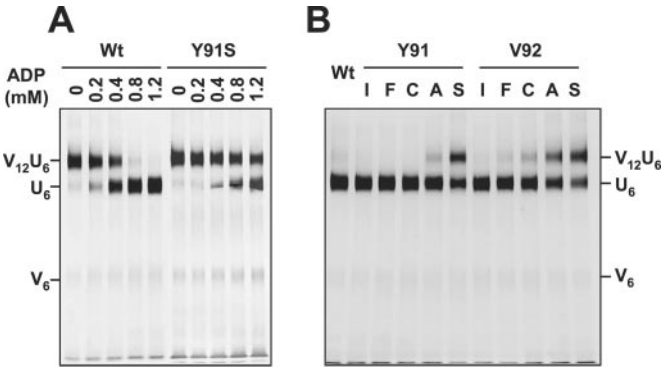

FIG. 7. **Effect of ADP on HslVU complex formation by HslU or its mutant forms with HslV.** *A*, reaction mixtures containing 0.13 μM wild-type HslU (*Wt*) or Y91S and 0.4 mM ATPγS were incubated with 0.18 μM HslV for 5 min at 37 °C in the presence of increasing concentrations of ADP. Cross-linking analysis was then performed by treatment with glutaraldehyde, followed by SDS-PAGE as described under "Experimental Procedures." *B*, HslU or its mutant forms (0.13 μM) were incubated with HslV (0.18 μM), 0.4 mM ATPγS, and 1 mM ADP for 5 min at 37 °C. The samples were then subjected to cross-linking analysis by incubation with glutaraldehyde. Proteins in the gels were visualized by silver staining.

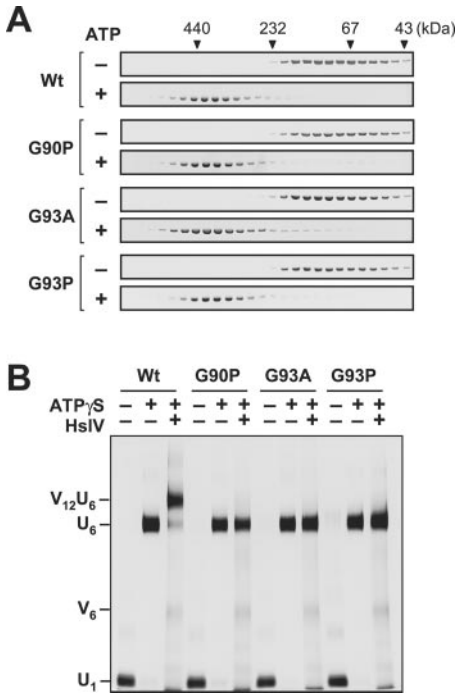

FIG. 8. **Oligomerization of the Gly^{90} and Gly^{93} mutant proteins and their ability to form HslVU complex with HslV.** *A*, HslU or its mutant forms (1.67 μM) were subjected to size exclusion chromatography as described under "Experimental Procedures." Prior to loading, the gel filtration column was equilibrated with 30 mM Tris-HCl (pH 7.6), 0.3 M NaCl, 1 mM EDTA, and 1 mM DTT in the absence (−) and presence of 1 mM ATP (+). Fractions (82 μl each) were collected and subjected to SDS-PAGE, followed by staining with Coomassie Blue R-250. The *arrowheads* indicate the fractions where the peaks of size markers eluted. *B*, HslU or its mutant forms (0.13 μM) were incubated in the absence or presence of HslV (0.18 μM), 1 mM ATPγS, or both for 5 min at 37 °C. The samples were then subjected to cross-linking analysis by incubation with glutaraldehyde. Proteins in the gels were visualized by silver staining.

activity. In addition, the hydrophobicity of the side chains, particularly of the 91st residue, appears to influence the affinity of ADP to HslU, because Y91C, unlike V92C, was much more sensitive to ADP than Y91S. However, we cannot exclude a possibility that the changes in the inhibitory effect of ADP on ATP hydrolysis could also be due to alterations in the binding affinity of ATP to HslU by the mutations, since the precise K_d values of ATP and ADP could not be determined.

Effects of GYVG Mutations on HslVU Complex Formation—ATPγS, a nonhydrolyzable ATP analog, supports the HslVU complex formation and the peptide hydrolysis by HslVU better than ATP (32). In contrast, ADP causes the dissociation of HslVU complex into HslV dodecamer and HslU hexamer and prevents the peptide hydrolysis (14, 31). To determine whether the HslU mutant proteins showing higher ATPase activity than wild-type HslU (*i.e.* Y91S, Y91A, V92S, V92A, and V92C) might have reduced affinities to ADP, and therefore their complexes with HslV show a lower tendency of dissociation than wild-type HslVU complex, we performed cross-linking analysis. Since ATP hydrolysis by HslU during incubation period for cross-linking markedly reduces the formation of HslVU complex, we used ATPγS instead of ATP. Among the mutant proteins, Y91S was first subjected to incubation with HslV and ATPγS in the presence of increasing concentrations of ADP for 5 min at 37 °C. The samples were then further incubated with glutaraldehyde and subjected to SDS-PAGE. Fig. 7*A* shows that wild-type HslVU complex dissociates into HslU and HslV at lower concentrations of ADP than Y91S/HslV complex, suggesting that Y91S has much lower affinity to ADP than wild-type HslU. We next carried out the same cross-linking analysis with all of the Tyr^{91} and Val^{92} mutant proteins as above but at a fixed ADP concentration. Y91A, V92S, V92A, and V92C were also more resistant from dissociation than wild-type HslU or the other mutant forms (Fig. 7*B*), again suggesting that they have lower affinity to ADP than wild-type HslU. Since the replacement of Tyr^{91} and Val^{92} by Ser or Ala showed little or no effect on the K_m values of ATP for HslU (data not shown), it is unlikely that the mutations alter the affinity of ATPγS to HslU. These results suggest that the increase in the ATPase activity of Y91S, Y91A, V92S, V92A, and V92C is at least in part due to their reduced affinity to ADP, which otherwise would exert the feedback inhibition on ATP cleavage. Thus, it is possible that the reduced affinity to ADP prevents the dissociation of HslVU complex into HslU and HslV, which in turn results in an increase in the HslV-mediated peptide hydrolysis.

Whereas G90P cleaved ATP although to a lesser extent than wild-type HslU, G93A and G93P showed little or no ATPase activity whether HslV was present or not (see Fig. 5*B*). We have previously shown that HslU behaves as monomers and dimers in the presence of NaCl but forms a hexamer upon incubation with ATP (32). Therefore, we first examined whether G93A, G93P, and G90P could bind ATP and form a hexameric complex in the presence of 0.3 M NaCl upon gel filtration analysis. Like wild-type HslU, all of the Gly mutant proteins that had been incubated with ATP eluted in the fractions corresponding to the size of HslU hexamer (Fig. 8*A*). On the other hand, the same proteins incubated without ATP behaved as monomer and dimmers. These results suggest that neither G93A nor G93P suffers from a gross structural defect at least in their abilities in ATP binding and hexamer formation.

We then examined whether the mutations might alter the ability of HslU to interact with HslV. G93A, G93P, and G90P were incubated with ATPγS in the absence and presence of HslV and then subjected to cross-linking analysis. Like wild-type HslU, both G93A and G93P could form a hexameric complex (Fig. 8*B*), again showing that they are capable of binding ATP. Unlike wild-type HslU, however, they were not able to form an HslVU complex with HslV. These results explain why the mutant proteins could not support the HslV-mediated proteolysis. Surprisingly, G90P neither could form a G90P/HslV complex in the presence of ATPγS, despite the finding that the mutant protein is capable of supporting the HslV-mediated

TABLE I
Ability of wild-type HslU and G90P in supporting the HslV-mediated peptide hydrolysis in the presence of ATP or ATPγS

Wild-type HslU and its mutant proteins (13.5 nM) were incubated with 4.4 nM HslV, 0.1 mM Z-Gly-Gly-Leu-AMC, and 1 mM ATP at 37 °C. Peptide hydrolysis was then assayed as described in Fig. 5. Similar data were obtained by three independent experiments.

HslU proteins	Peptide hydrolysis by HslV with	
	ATP	ATPγS
	%	
Wild-type HslU	100	184
G90P	59	14

peptide hydrolysis although about 60% as well as wild-type HslU in the presence of ATP. Therefore, we compared the ability of G90P to that of wild-type HslU in supporting the peptide hydrolysis by HslV in the presence of ATPγS and ATP. In accord with our previous observation (31), wild-type HslU in the presence of ATPγS supported the HslV-mediated peptide hydrolysis about twice as much as in the presence of ATP (Table I). In contrast, G90P in the presence of ATPγS could support the peptidase activity less than 25% of that seen with ATP. Thus, it appears that ATPγS-bound G90P may interact with HslV much less tightly than ATP-bound form.

DISCUSSION

The present studies have demonstrated that the GYVG^{93} pore motif of HslU plays important roles in unfolding and translocation of protein substrates for their degradation at the inner proteolytic chamber of HslV. The degradation of SulA required the flexibility of Gly^{90} and the aromatic ring structures of the 91st amino acid, suggesting that these structural features of the pore motif are essential at least for the step involving unfolding of natively folded proteins. In addition to these structural features, the flexibility of Gly^{93} might also be required for unfolding step, since it is strictly conserved in the ATPase components of all ATP-dependent proteases. On the other hand, the structural features that are needed for SulA degradation were not stringently required for the degradation of casein, suggesting that they are not essential for the translocation of unfolded proteins. Nevertheless, the same features were highly preferred for hydrolysis of the unfolded protein. Thus, unfolding and translocation may occur as a coupled process rather than separate steps, as suggested by the reported studies with ClpAP (33, 34). In contrast, none of the structural features of the GYVG motif in HslU was required for the HslV-mediated peptide hydrolysis, consistent with an expectation that small substrates would need neither unfolding nor translocation process.

A number of recent reports have suggested the importance of pore motif of Clp/Hsp100 and AAA family members in binding, engagement, unfolding, and/or translocation of protein substrates. FtsH has Phe^{228} at the position that corresponds to Tyr^{91} in the GYVG pore motif of HslU. Yamada-Inagawa *et al.* (35) have demonstrated that FtsH having any of the large hydrophobic amino acids (*e.g.* Leu, Ile, or Val) or an aromatic amino acid (*e.g.* Tyr or Trp) in place of Phe^{228} is capable of degrading of σ^{32} *in vivo*. This nonstringent requirement of FtsH for degradation of σ^{32} is in marked contrast with HslU that strictly requires a ring structured aromatic amino acid at the 91st position to support the HslV-mediated degradation of SulA. Herman *et al.* (36) have shown that FtsH lacks robust unfoldase activity and degradation by FtsH occurs efficiently only when the substrate is a protein of low intrinsic thermodynamic stability. Thus, it may be possible that Phe228 in the pore motif of FtsH may play a role in translocation of unfolded proteins but not necessarily in unfolding of native proteins.

ClpX ATPase is a hexamer that has the same GYVG pore motif as HslU ATPase. Siddiqui *et al.* (37) have recently shown that the replacement of Val154 by Phe causes a severe defect in the ability of ClpX in supporting the activity of its cognate ClpP peptidase against certain substrates (*e.g.* Arc-ssrA) but has little effect on that against other substrates (*e.g.* IscS-Arc). They further showed that the defect in the ability of V154F in supporting the degradation of Arc-ssrA was due to a specific defect in the binding of the protein substrate. Based on these observations, they suggested that the ClpX pore plays a role in differential recognition of protein substrates. Of note is our finding that the replacement of Val^{92} (corresponding to Val^{154} in ClpX) by Phe abolished the ability of HslU in supporting the SulA degradation by HslV. This inability of V92F in supporting the SulA degradation may also be due to its defect in binding to the substrate. Thus, it is possible that V92F may be capable of binding to other unidentified substrates for degradation by HslV.

ClpB is a molecular chaperone that cooperates with the DnaK-DnaJ-GrpE chaperone system in solubilization and refolding of aggregated proteins (38–40). ClpB has two conserved aromatic residues, Tyr^{51} and Tyr^{653}, positioned at the central pores of AAA domains. Recently, it has been shown that both of the aromatic residues play a critical role in direct binding of aggregated proteins and their translocation (41–43). Thus, lines of evidence indicate that the pore motif of Clp/Hsp100 and AAA family members play an essential role in most, if not all, processes of protein binding, engagement, unfolding, and translocation.

A data base search (35) has revealed that 68% of proteins that belong to Clp/Hsp100 and AAA family have Tyr, and 23% of them have Phe or Trp at the position that corresponds to Tyr^{91} in the GYVG motif of HslU. This statistics clearly suggests the importance of aromatic ring structure in the common function of the primary pore motif. Then, why should it be an aromatic amino acid? The answer may lie in the capability of aromatic rings to constitute unique noncovalent interaction with other molecules: cation-π and π-π interactions (44, 45). These interactions have been estimated to play substantial roles in protein-ligand binding, enzyme-substrate interaction, and protein folding (44, 46). Especially for cation-π interaction, it is known that aromatic residues can attract positively charged amino group of Lys or guanidinium group of Arg in significant magnitude (47). The π-π interaction between benzene rings of two Phe residues has also been estimated to contribute an attractive force, depending on the geometric orientation of the aromatic rings (48). Consistently, Schlieker *et al.* (41) have revealed that ClpB binds to peptides rich in basic and aromatic amino acids with strong preference. Thus, it appears that the aromatic amino acids are stringently conserved in the pore motif for their essential function in protein binding, unfolding, and translocation by adapting the cation-π or π-π interaction with the substrates.

Noteworthy was the finding that G93A and G93P were not capable of supporting the proteolytic activity of HslV against any of the substrates tested. Moreover, they showed little or no ATPase activity and neither could form an HslVU complex with HslV, despite the fact that they could bind ATP and form a hexameric complex as well as wild-type HslU. It is also of note that G90P interacts with HslV and supports the peptide hydrolysis by HslV in the presence of ATP but not in the presence of ATPγS, which is known to promote the interaction of wild-type HslU with HslV and the peptide hydrolysis by HslVU complex even better than ATP (31). We have previously shown that ATP binding, but not its hydrolysis, is required for the oligomerization of HslU, which is essential for its interaction

with HslV (31). It has also shown that the highly conserved HslU C terminus is inserted at the HslV-HslV subunit interface when ATP is bound, otherwise it is buried at the HslU-HslU subunit interface (11, 17, 49, 50). In addition, deletion of 7 amino acids from the C terminus prevents the interaction of HslU with HslV, indicating the importance of the C-terminal region in the formation of HslVU complex (25). Taken together, it may be possible that binding of ATP to G93A and G93P or that of ATPγS to G90P induces a conformational change sufficient for the interaction between subunits for hexamer formation, but this change may not be further transmitted to the C-terminal tail region.

Of interest was the finding that mutations in the pore motif showed a marked change in the ability to HslU in ATP hydrolysis. Particularly, the substitution of Tyr^{91} or Val^{92} with Ala or Ser increased both the basal and HslV-stimulated ATPase activities of HslU more than 3-fold. This increase in the ATPase activity of the HslU mutant proteins appears to be at least in part attributed to the decrease in their affinity toward ADP, since the inhibitory ADP could be released from the ATPase at a faster rate. The increase in the ATPase activity could also be attributed to the increase in the motion of the pore motif upon the replacement of Tyr^{91} or Val^{92} by Ala or Ser. Structural analyses by x-ray crystallography have shown that the cycle of ATP hydrolysis accompanies a movement of the central pore region of HslU (16, 17). Therefore, the removal of the bulky hydrophobic side chains from the sterically crowded pore region of HslU might facilitate the motion of the pore motif and in turn promote the cycle of ATP hydrolysis. This notion is further supported by our findings that the elimination of flexibility, thus preventing the movement of the pore motif by the replacement of Gly^{93} by Pro, results in complete loss of the HslU ATPase activity. Thus, the local structure and/or dynamics of the pore motif may affect the ATPase activity of HslU through a bidirectional coupling mechanism, as was suggested by the work with FtsH (35).

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