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Stepwise Activity of ClpY (HslU) Mutants in the Processive Degradation of *Escherichia coli* ClpYQ (HslUV) Protease Substrates▽

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In *Escherichia coli*, ClpYQ (HslUV) is a two-component ATP-dependent protease composed of ClpY (HslU), an ATPase with unfolding activity, and ClpQ (HslV), a peptidase. In the ClpYQ proteolytic complex, the hexameric rings of ClpY (HslU) are responsible for protein recognition, unfolding, and translocation into the proteolytic inner chamber of the dodecameric ClpQ (HslV). Each of the three domains, N, I, and C, in ClpY has its own distinct activity. The double loops (amino acids [aa] 137 to 150 and 175 to 209) in domain I of ClpY are necessary for initial recognition/tethering of natural substrates such as SulA, a cell division inhibitor protein. The highly conserved sequence GYVG (aa 90 to 93) pore I site, along with the GESSG pore II site (aa 265 to 269), contribute to the central pore of ClpY in domain N. These two central loops of ClpY are in the center of its hexameric ring in which the energy of ATP hydrolysis allows substrate translocation and then degradation by ClpQ. However, no data have been obtained to determine the effect of the central loops on substrate binding or as part of the processivity of the ClpYQ complex. Thus, we probed the features of ClpY important for substrate engagement and protease processivity via random PCR or site-specific mutagenesis. In yeast two-hybrid analysis and pulldown assays, using isolated ClpY mutants and the pore I or pore II site of ClpY, each was examined for its influence on the adjoining structural regions of the substrates. The pore I site is essential for the translocation of the engaged substrates. Our *in vivo* study of the ClpY mutants also revealed that an ATP-binding site in domain N, separate from its role in polypeptide (ClpY) oligomerization, is required for complex formation with ClpQ. Additionally, we found that the tyrosine residue at position 408 in ClpY is critical for stabilization of hexamer formation between subunits. Therefore, our studies suggest that stepwise activities of the ClpYQ protease are necessary to facilitate the processive degradation of its natural substrates.

ClpYQ (also known as HslUV) along with Lon and ClpAP are the three primary ATP-dependent proteases in *Escherichia coli*, responsible for 70% to 80% of protein degradation *in vivo* (12, 29). ClpYQ (HslUV) is found in 60% of all eubacteria and is the only ATP-dependent protease for which the entire structure is known (3, 5, 48). The Clp (caseinolytic protease) ATPases belong to the AAA^+ (ATPases associated with diverse cellular activities) superfamily of proteins, and ClpY is among the best understood. Unlike other two-component Clp proteases in which different proteolytic cores can interact with multiple types of different chaperones (unfoldases), e.g., ClpP with ClpA or ClpE, ClpC, or ClpX, ClpQ interacts singularly with the ClpY chaperone (21, 30).

The genes encoding ClpYQ (HslUV) are part of a heat-shock operon (6, 28) in which the first gene encodes ClpQ (19 kDa), a small-subunit peptidase, and the second gene encodes the large subunit ClpY (49 kDa) (17, 32, 38, 49). Each ClpQ or ClpY monomer self-oligomerizes as a hexamer, and four hexamers constitute a dumbbell-shaped complex ($Y^6Q^6Q^6Y^6$) (17, 38). ClpY transfers substrates from outside the hexameric cylinder into the catalytic core, wherein ClpQ degrades the substrates (17, 32, 38, 49). In the ClpYQ complex, ClpY has an ATPase activity (17, 32, 38, 49).

Crystallography indicates that ClpY is partitioned into three domains, N, I, and C (derived from 1E94 [Protein Data Bank], using the PyMOL program) (Fig. 1A) (3, 7, 41). In its tertiary structure, the N domain contains an ATP pocket with its ATPase activity and domain I protrudes outward to allow gripping of the substrates (44, 45), while a C-terminal tail is necessary for activation of ClpQ (Fig. 1A) (36, 39). The ATP-bound hexameric ClpY is required for substrate recognition (Fig. 1B) (5). Four proteins, SulA, RcsA, RpoH, and TraJ, are natural substrates of ClpY (14, 15, 16, 18, 22, 24, 47). The biological function, biochemical activity, X-ray crystal structure, and evolutionary role of the ClpYQ protease were recently reviewed (46).

The asymmetric hydrolysis of three or four ATPs in the ClpY hexamer is indispensable to the binding, unfolding, translocation, and substrate degradation by ClpQ (48). All six active sites of ClpQ are required for the degradation of the substrates (25). Two central loops of ClpY, the pore I site (amino acids [aa] ~90 to 93; GYVG) (Fig. 1A) and the pore II site (aa ~265 to 269; GESSG) (Fig. 1A) in domain N, are in

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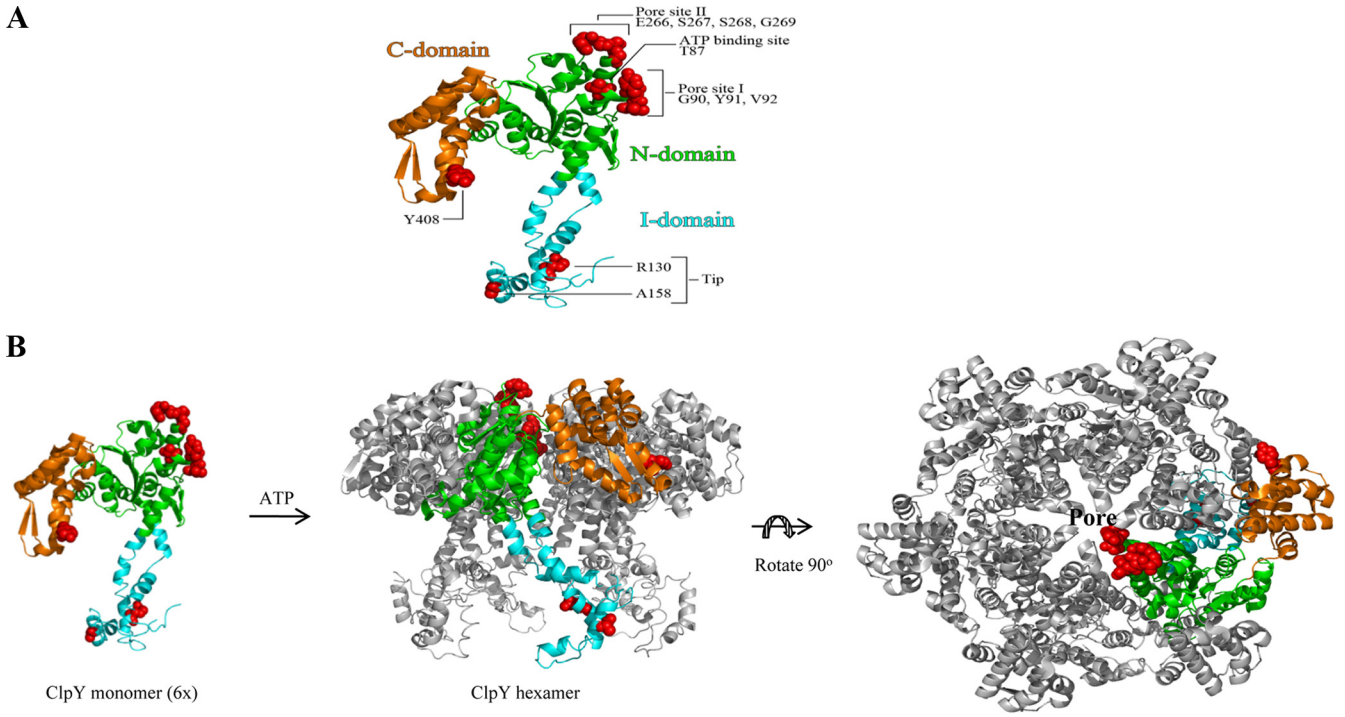

C ClpY mutants used in this study:

ATP-binding site	Pore I				tip	Pore II			Domain C
[a]T87I	[a]G90D	[b, c]Y91F	[b, c]Y91S	[b, c] V92F	[a]R130H, A158T	[b]E266Q	[b] S267L	[b] S268L	[a] G269C	[b]Y408A

FIG. 1. Domains of ClpY, its functional motifs, and mutations used in this study. (A) The crystal structure of ClpY. It contains three domains, N, I, and C, which are a total of 443 aa. The N domain (aa 2 to 109 and aa 244 to 332) (green) includes the Walker BoxA, or P loop (aa 57 to 66), and BoxB (aa 253 to 256), the nucleotide binding pocket (aa 17 to 19, aa 57 to 66, and aa 80 to 89), and pore I (aa 89 to 94) and pore II (aa 265 to 269) sites; domain I (aa 110 to 243) (blue) is protruding out with the I tip (aa 154 to 165); domain C (aa 333 to 443) (brown) is for subunit-subunit interaction; the specific amino acid(s), which is designated and labeled with a red ball, was thereafter studied in this work. (B) The ClpY monomer, in the presence of ATP, forms a hexamer, and the side or top view of the ClpY hexamer was shown with the specific amino acid(s) (red) described above in one subunit (color shades). (C) The isolated ClpY mutants as indicated, with a mutation(s) in the specific site(s) described above. a, mutants isolated by random mutagenesis (27). b, mutants isolated by site-directed mutagenesis. c, mutants also isolated by Park et al. (35).

the center of its hexameric ring (44, 45, 50). *In vitro*, ClpY pore I mutants were defective in substrate translocation in the presence of ClpQ (35). Yeast two-hybrid analysis detected the ability of ClpY to bind to its natural substrate SulA (26), and surface plasmon resonance (SPR) analysis detected its ability to associate *in vitro* with the MBP-SulA fusion protein (2). The double loops (aa 137 to 150 and 175 to 209) in domain I of ClpY function primarily in the initial recognition/tethering of its natural substrates (27).

It has been proposed that ClpY carries the tethering site (T) and the pore site (P) that are useful in binding to an artificial Arc substrate for degradation in the ClpYQ complex (43). Based on the crystal structure (41), the multiscale molecular dynamic simulations elicited a model in which a paddling mechanism translocates the substrate (a threaded single polypeptide) of an ATP-bound ClpY hexamer; i.e., Tyr-91S in ClpY, with open-up (OU) and closed-down (CD) forms, that in turn grips a substrate and with an upward motion unidirectionally translocates the substrates (19). In addition, the unfolding and translocation of the substrate protein (four-helix bundle protein) by the central channel loop structure of ClpY with the repetitive allosteric ATPase cycle-driven mechanism has been recently described in coarse-grained molecular simulations (20). However, the functional roles of the amino acid residues that form the pore I or pore II site of ClpY and are implicated in binding to nearby substrate structural regions during processive degradation remain to be elucidated.

Hence, in this study, we demonstrated that the pore I site of ClpY has an effect on the adjoining structural region in protein substrates, and the pore I site is essential for the translocation of substrates. The pore II site also interfaces with nearby regions in the substrates but is not necessary for their translocation. Our *in vivo* study of the ClpY mutants also revealed that an ATP-binding site in domain N, separate from its role in polypeptide (ClpY) oligomerization, is required for complex formation with ClpQ. Therefore, our studies suggest that stepwise activities of ClpYQ protease are necessary to facilitate the processive degradation of its natural substrates. Our study in-

TABLE 1. The strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Yeast strain		
EGY48	MAT**a** *his3 trp1 ura2* $LexA_{op(x6)}$-*LEU2* p8op-*lacZ*	Clontech
Bacterial strains		
AC3112	*lon clpQY cpsB*::*lacZ*	21
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lon*; λ phage carrying the T7 RNA polymerase	Novagen
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Novagen
Plasmids		
pGilda	Amp^r; *HIS*$^+$; BD[a] domain gene	Clontech
pB42AD	Amp^r; *TRP*$^+$; AD[b] domain gene	Clontech
pTH18kr	Kan^r; *ori*(pSC101); P_{tac} promoter	8
pBAD33	*ori*(pACYC); *araC* P_{BAD}; Chl^r	7
pBAD24	*ori*(pBR322); *araC* P_{BAD}; Amp^r	7
pET21a	Amp^r; T7 promoter	Novagen
pMAL-p-SulA	*malE-sulA*$^+$ fusion gene in the pMAL plasmid	9
pGilda-*sulA*$^+$	BD-*sulA*$^+$ fusion gene in pGilda	25
pGilda-*sulA* (*M89I*)	BD-*sulA*(*M89I*) fusion gene in pGilda	25
pB42AD-*clpY*$^+$	AD-*clpY* $^+$ fusion gene in pB42AD	25
pB42AD-*clpY* derivatives	AD-*clpY* mutation(s)[c] fusion gene in pB42AD	This work
pBAD33-*clpQ*$^+$	*clpQ*$^+$ in pBAD33	26
pTH18kr-*ha-sulA*$^+$	*ha-sulA*$^+$ under the P_{tac} promoter	26
pBAD24-*clpY* derivatives	*clpY* mutation(s)[c] in pBAD24	This work
pET21a-(6×His)-*clpQ*$^+$	6×His-tagged *clpQ*$^+$ in pET21a	This work
pET21a-(6×His)-*clpY*$^+$	6×His-tagged *clpY*$^+$ in pET21a	This work
pET21a-(6×His)-*clpY* derivatives	6×His-tagged *clpY* mutation(s)[c] in pET21a	This work

[a] BD, BD domain encoded by the *lexA* DNA-binding domain gene.
[b] AD, B42 polypeptide activation domain gene encoding the AD domain plus the *ha* epitope tag.
[c] Various mutation(s) in the gene *clpY*, with the encoded mutants shown in Fig. 1C.

dicates that ClpY recognizes its substrates with an initial tethering and that the role of pore site I is to mediate substrate binding for the subsequent translocation into the core site for degradation by ClpQ. Moreover, ClpY (T87I), with a mutation in the ATP-binding site, did not undergo a conformational change to its hexameric form for association with ClpQ. Tyrosine residue 408 in ClpY, like residue 385 in ClpX (13), was necessary for self-oligomerization, and this activity is likely important for *in vivo* protein-subunit stability.

MATERIALS AND METHODS

Strains and plasmids. All the strains and plasmids used in this study are listed in Table 1. *Saccharomyces cerevisiae* strain EGY48 [*MAT***a** *his3 trp1 ura2* $lexA_{op(x6)}$-*LEU2*; op(X6) indicates six copies of the operator site] was acquired from Clontech (Palo Alto, CA). EGY48 carries the plasmid p8op-*lacZ* (with the *ura*$^+$ gene) and a $lexA_{op(x6)}$-*LEU2* fusion integrated into the chromosome. pGilda, a LexA DNA binding domain (BD) vector, and pB42AD, a B42 polypeptide activation domain (AD) vector with a hemagglutinin (HA) epitope tag, were obtained from Clontech. Plasmid pTH18kr (9) was obtained from the National BioResource Project (NBRP) (1). Strain AC3112 (*lon* Δ*clpQY cpsB*::*lacZ*) was used as a host for the methyl-methane-sulfonate (MMS) test and for β-galactosidase assays of *cpsB*::*lacZ* expression. The plasmids pB42AD-*clpY*$^+$, pGilda-*sulA*$^+$, pGilda-*sulA*(*M89I*), and pTH18kr-*sulA*$^+$ were constructed as described previously (26, 27). Plasmids pBAD33 and pBAD24 were used to clone wild-type and mutant genes, which were thus subject to regulation by arabinose induction and glucose repression (8). Expression of HA-SulA from the plasmid pTH18kr-HA-*sulA*$^+$ was subject to isopropyl-β-D-thiogalactopyranoside (IPTG) induction (9).

Media and reagents. For yeast growth, synthetic dropout (DO) minimal medium (SD) was purchased from Clontech. DO supplements containing nucleotides and amino acid residues were prepared according to the manufacturer's instructions. Minimal media with different sugars were prepared as described by Miller (31). *E. coli* was grown in Luria broth (LB). The supplements were as follows: 2% glucose (Glc), 2% galactose (Gal), and 1% raffinose (Raf), and 100 μg/ml ampicillin (Amp), 25 μg/ml kanamycin (Kan), 34 μg/ml chloramphenicol (Chl), or 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), as indicated.

Random mutagenesis of ClpY. Oligonucleotide primers were used for error-prone PCR mutagenesis, and ClpY mutants were identified as described previously (27). Mutagenized PCR products encompassing the internal region of ClpY (from aa 63 to aa 282) were designed to allow homologous recombination, following cotransformation with linearized pB42AD-*clpY*$^+$ (cut with NruI in the *clpY* gene) into *S. cerevisiae* strain EGY48 carrying pGilda-*sulA*$^+$. Recombinants were selected by growth on SD-Trp-His-Ura, since pB42AD carries the Trp$^+$ marker. The resulting transformants were then screened on X-Gal minimal medium containing raffinose and galactose. Light or dark blue colonies were saved for further plasmid isolation. The extracted plasmids were then transformed into KC-8, and Trp$^+$ transformants were selected on glucose minimal (GM) medium plus DO in the absence of tryptophan. Plasmid pB42AD carrying *clpY*$^+$ and its derivatives was recovered from Trp$^+$ colonies and transformed back into strain EGY48(p8op-*lacZ*) carrying pGilda-*sulA*$^+$. Plasmid phenotypes were verified again by plating the transformants on X-Gal or Leu-depleted medium. DNA sequence analysis was used to identify plasmids containing mutations in *clpY*.

Derivatives of the yeast strain EGY48(p8op-*lacZ*) coexpressing BD and AD hybrid proteins were assayed for reporter gene expression on X-Gal plates and in *Leu*$^+$-selective assays as previously described.

Site-directed mutagenesis for construction of *clpY*(*Y91F*), *clpY*(*Y91S*), *clpY*(*V92F*), *clpY*(*E266Q*), *clpY*(*S267L*), *clpY*(*S268L*), and *clpY*(*Y408A*). A two-step PCR analysis was used to construct *clpY*(*Y91F*), *clpY*(*Y91S*), *clpY*(*V92F*), *clpY*(*E266Q*), *clpY*(*S267L*), *clpY*(*S268L*), and *clpY*(*Y408A*). EcoRI-BamHI DNA fragments containing the mutated genes were each constructed using two sets of primers. For example, to construct *clpY*(*Y91F*), the first-step primers F1 (5′-GCGGAATTCATGTCTGAAATGACC-3′) and R1 (5′-CACTTCCTTACCGACG**A**AGCCCACTTCGGTGAA-3′) were used to yield a left-half fragment containing the mutagenized site (bold); similarly, the primers F2 (5′-TTCACCGAAGTGGGC**TT**CGTCGGTAAGGAAGTG-3′) and R2 (5′-GCGGGATCCTTATAGGATAAAACG-3′) were used to yield a right-half fragment that overlapped the mu-

tagenized site (restriction enzyme sequences are underlined). The resulting overlapping fragments were then used as templates, with F1 and R2 as primers, for synthesis of the final product, which was inserted between the EcoRI and BamHI sites of pGilda. Other ClpY mutants were also constructed by using the procedure described above. However, *clpY*(*Y408A*) is a derivative of *clpY*$^+$ (SmaI) (27). Thus, the plasmids pGilda-*clpY*(*E266Q*), pGilda-*clpY*(*S267L*), pGilda-*clpY*(*S268L*), and pGilda-*clpY*(*Y408A*) were constructed by cloning the *clpY*(*E266Q*), *clpY*(*S267L*), *clpY*(S268L), and *clpY*(*Y408A*) genes, respectively, into pGilda at the EcoRI and BamHI sites. The plasmids pB42AD-*clpY*(*E266Q*), pB42AD-*clpY*(*S267L*), pB42AD-*clpY*(*S268L*), and pB42AD-*clpY*(*Y408A*) were constructed by cutting the corresponding DNA fragments of *clpY*(*E266Q*), *clpY*(*S267L*), *clpY*(*S268L*), and *clpY*(*Y408A*) from pGilda-*clpY*(*E266Q*), -*clpY*(*S267L*), -*clpY*(*S268L*), and -*clpY*(*Y408A*) and ligating each DNA fragment into pB42AD between the EcoRI and XhoI sites. The newly constructed plasmids were all sequenced to confirm their correct construction. By cutting the pB42AD-*clpY* $^+$ and its mutant derivatives in plasmids with EcoRI and HindIII, the *clpY*$^+$ and its mutant derivatives were subcloned into pBAD24 at the identical compatible sites and the resulting plasmids were designated pBAD24-*clpY*$^+$, etc., in series.

MMS test and β-galactosidase assays of *cpsB*::*lacZ* expression. Assays for *cpsB*::*lacZ* expression and MMS tests in bacteria were performed as described previously (27). Strain AC3112 (*lon*, *clpQ*, and *clpY*, mutants and *cpsB*::*lacZ*) (22) carrying plasmids pBAD33-*clpQ*$^+$ and pBAD24-*clpY* $^+$ or its derivatives were grown overnight in LB medium plus ampicillin (50 μg/ml), chloramphenicol (12.5 μg/ml), and glucose (1%) or arabinose (0.5%). The overnight cultures were inoculated at a 1/100 dilution into similar media and grown to log phase. Efficiency of plating (EOP) in the presence of MMS was calculated as follows: the titers of colonies on LB plus 0.5% arabinose agar media with MMS divided by the corresponding titers on LB plus 0.5% arabinose in the absence of MMS. β-Galactosidase activity assays were performed in triplicate three times (31).

Western blot assays for detection of HA-SulA, ClpQ, and ClpY derivatives from bacterial cells. HA-SulA, ClpQ, and ClpY, as well as its derivatives, were detected as previously described (28). In plasmid pTH18kr-*ha-sulA*$^+$, *ha-sulA*$^+$ expression is directed by the *tac* promoter, and the host AC3112 carries *lacI*^{q}. AC3112 cells containing pBAD33 (Chl^r), pBAD24 (Amp^r), and pTH18kr-*ha-sulA*$^+$ (Kan^r) were used as controls. AC3112 cells containing pTH18kr-*ha-sulA*$^+$, pBAD33-*clpQ*$^+$, and either pBAD24-*clpY* $^+$ or its derivatives were grown overnight on LB media with 0.5% arabinose and the appropriate antibiotics. Dilutions (1:100) of the overnight bacterial cultures were inoculated into identical fresh media, which were incubated with shaking at 30°C. When the cultures reached an approximate optical density at 600 nm (OD_{600}) of 0.5, 100 mM IPTG was added to a final concentration of 1 mM. After 3 h, the cell extracts were prepared by sonication, dissolved in 2× sample buffer, and analyzed on 12.5% SDS-polyacrylamide gels. Western blots were performed using monoclonal HA antibody (Roche) to detect HA-SulA and multiserum ClpQ and ClpY antibodies for detection of the corresponding proteins. Western blots were developed by using enhanced chemiluminescence (Pierce).

His tag affinity purification of ClpQ, ClpY, and ClpY derivative mutants. Primer sequences for creation of 6×His-tagged ClpQ were as follows: 5′-GGA ATTCCATATGCACCACCACCACCACCACACAACTATAGTAAGCGTA C-3′ and 5′-CCCAACTTGGGTTAGTGGTGGTGGTGGTGGTGCGCTTTG TAGCTTAATTC-3′. The PCR product was digested with NdeI and HindIII and then ligated into pET21a. For 6×His-CplY, PCR amplification was performed using pB42AD-*clpY*$^+$ as the template and primers 5′-GGGTTTCATATGCAC CACCACCACCACCACTCTGAAATGACCCCACGC-3′ and 5′-CCAAGCTT GGTTATAGGATAAAACGGCTCAGA-3′. The resulting 6×His-*clpY*$^+$ was cloned into the NdeI-HindIII site of pET21a(+) (Novagen). The resultant pET21a-6×His-*clpY*$^+$ was transformed into *E. coli* BL21(DE3). Other His-ClpY derivatives were constructed by similar methods, using its derivative mutants as templates. Cells carrying pET21a-*clpY*$^+$ and its derivatives were collected after a 3-h induction with 1 mM IPTG at 37°C. After centrifugation, cell pellets were resuspended in native buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, pH 7.5) and lysed by sonication. The lysates were centrifuged at 4°C, and the supernatants were collected and transferred to TALON metal affinity resin columns (Clontech, CA). The input supernatant was drained by gravity flow. Five- to 10-bed volumes of equilibration/wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0) were added to the resin column for the washing step. After washing, elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 7.0) was then added and the eluent was collected. All the purified proteins were subsequently analyzed on SDS-PAGE gels.

Purification of the MBP-SulA fusion protein. *E. coli* DH5α cells containing the plasmid pMAL-p-SulA, which were a gift from Atsushi Higashitani (10), were cultured in LB medium containing 0.2% glucose (200 ml) at 30°C. Cultures were grown to an OD_{600} of 0.5, and IPTG (final concentration, 0.3 mM) was added for induction of MBP-SulA. After 4 h, cells were harvested by centrifugation at 5,000 × *g* for 20 min and then suspended in column buffer (20 mM HEPES, 200 mM NaCl, 1 mM EDTA, pH 7.4). After sonication, the suspension was centrifuged at 12,000 × *g* for 30 min. The supernatant was loaded onto amylose resin (New England BioLabs) and washed with the column buffer (12-bed-column volumes). MBP-SulA was eluted by elution buffer (column buffer plus 10 mM maltose). The protein concentration was determined by using the Bradford method with bovine serum albumin as the standard (4).

Pulldown assays. The protein affinity pulldown analyses as described previously (34) were modified for use in the following procedure. His-ClpY or its derivative mutants (6-mer; 1.3 μM) and MBP-SulA (7.8 μM) in 50 μl with 50 mm HEPES buffer (pH 8) with 150 mm NaCl, 5% glycerol, and 0.04% Triton X-100 were incubated at 37°C for 3 h in the absence or presence of 5 mM ATP and 5 mM $MgCl_2$. After incubation, the mixtures were supplemented with 20 μl amylose resins (NEB) and rocked at 4°C for at least 3 h. The resins were washed 10 times with 0.2 ml 50 mM HEPES buffer (pH 8) with 300 mm NaCl, 5% glycerol, 0.04% Triton X-100, and 5 mM $MgCl_2$. Proteins bound to amylose resins were eluted by 20 μl SDS sampling buffer, subjected to SDS-PAGE, and stained with Coomassie blue R-250. In addition, the portions of pulldown samples were independently detected with Western blot analysis using ClpY antibody and MalE antibody.

Degradation of MBP-SulA by ClpY, ClpY(T87I), and ClpY(Y408A) in the presence of ClpQ. The degradation of MBP-SulA was assayed as previously described (36). Reaction mixtures (60 μl) containing 1.6 μM MBP-SulA, 6.62 μM ClpQ, and 4.05 μM ClpY or its mutant derivatives in 0.1 M HEPES buffer (pH 8), 10 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol (DTT), and 1 mM EDTA were incubated for various times at 37°C. After incubation, reactions were stopped by addition of 30 μl of 0.75 mM Tris-HCl (pH 6.8) containing 7.5% SDS and 10% (vol/vol) 2-mercaptoethanol. The samples were then subjected to SDS-PAGE on 12% slab gels, followed by staining of the gels with Coomassie blue R-250. The percentage of MBP-SulA remaining after degradation was analyzed by ImageJ (version 1.45d) from the National Institutes of Health (NIH) (37). Basically, ImageJ was used to open the saved image file and an appropriate image type was chosen by selecting "Image" in the tool box. By selecting "Analyze-Gel," the protein bands in gel were rectangularly selected and the identical rectangular box was dragged for each sample in various lanes and labeled appropriately. The protein stable across each lane was regarded as an internal control. Each band was plotted as a peak, using "tool 4" to close off the bottom and "tool 8" to integrate and quantify each area as described in the manual instructions (37). The measurement of each sample was performed three times for the triplicate data. The percentages of the remaining substrates in assays were calculated using the sample taken at time zero as 100%, and the standard deviation (SD) was determined.

Cross-linking analysis of ClpYQ complexes. Purified ClpY (6-mer; 4 μM) or its derivatives and ClpQ (12-mer; 4 μM) were mixed in 0.1 M HEPES buffer (pH 8) containing 10 mM $MgCl_2$, 1 mM ATP-γS, 1 mM dithiothreitol, 1 mM EDTA. The mixtures were preincubated for 5 min at 37°C. Glutaraldehyde (0.4% [vol/vol]) was added in a total volume of 40 μl after incubation for 20 min at 37°C, and then the samples were mixed with 30 μl 0.75 M Tris-HCl (pH 6.8) containing 7.5% SDS and 10% 2-mercaptoethanol. The samples were finally subjected to SDS-PAGE on a 4 to 12% gradient gel.

Isothermal titration calorimetry. The binding thermodynamics of ClpY and ClpY(T87I) with ATP-γS were measured using an iTC200 system (MicroCal). ClpY or ClpY(T87I) [200 μl, 25 μM, 6-mer–ClpY or 6-mer-ClpY(T87I) in buffer] in the isothermal calorimetric cell was titrated with 750 μM ATP-γS dissolved in the same buffer in a 40-μl injector syringe at 25°C. The injector syringe was programmed to titrate ATP-γS into the cell with 1.9 μl per injection at 150-s intervals. The stirring rate of the injector was 1,000 rpm. As a control, ClpY or ClpY(T87I) was injected into buffer under the same conditions. The raw calorimetry data were collected and analyzed using Origin version 7.0 data analysis software (MicroCal). The binding isotherms were fitted to the one-set-of-sites binding model, giving values for the changes in enthalpy (ΔH) and entropy (ΔS) of binding. The Gibbs free energy of binding, ΔG, was also calculated using the following equation: $\Delta G = -RT\ln(1/K_d) = \Delta H - T\Delta S$, where K_d is the dissociation constant, R is the gas constant, and T is absolute temperature.

ATPase assays of ClpYQ and its mutant derivatives in the presence of MBP-SulA. ATP hydrolysis was measured using a coupled assay (33). The reaction mixtures contained 2.5 mM ATP, 0.375 mM NADH, 1.0 mM phosphoenolpyruvate, 5.0 U/ml lactate dehydrogenase (Sigma), and 5.0 U/ml pyruvate kinase (Sigma) in HEPES buffer. ClpY (6-mer; 8 μM), ClpQ (12-mer; 8 μM), and MBP-SulA (4.9 μM) were added in series into the mixture and then incubated at 37°C for 2 h; the decrease of NADH was determined by measurement of fluo-

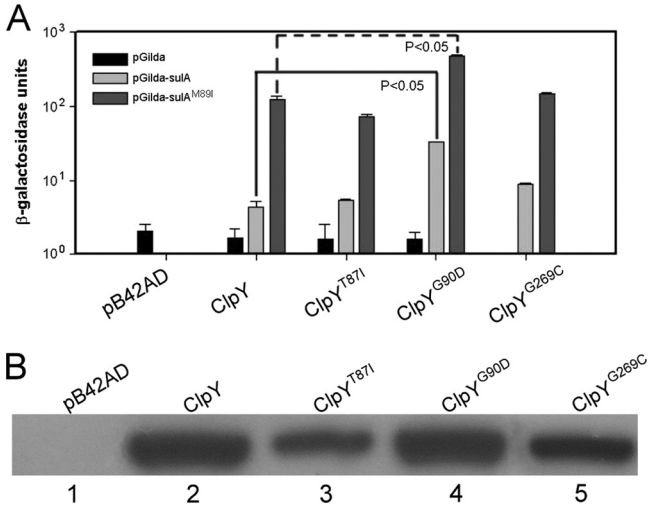

FIG. 2. Expression of *lacZ* and LEU2 in the yeast strain EGY48 (p8op-*lacZ*). Each strain contains plasmids encoding a pair of AD and BD fusion proteins. The BD plasmids are derived from pGilda, and the AD plasmids are derived from pB42AD. (A) β-Galactosidase activities of EGY48 carrying AD-ClpY derivatives together with BD-SulA or BD-SulA(M89I). Error bars indicate standard deviations for three assays of each strain. $P < 0.05$ was considered statistically significant with Student's *t* test. Bracket lines indicate the binding affinity of ClpY mutants, significantly different from that of the wild-type ClpY. (B) Western blots of ClpY and its derivatives in the BD fusion protein analyzed using an anti-LexA antibody.

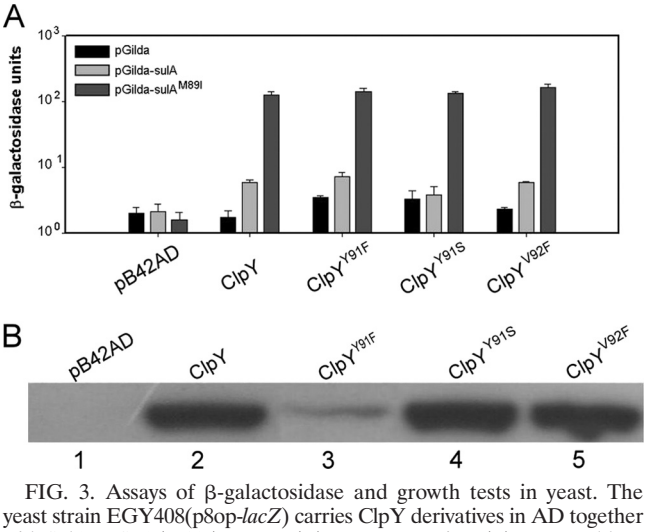

FIG. 3. Assays of β-galactosidase and growth tests in yeast. The yeast strain EGY408(p8op-*lacZ*) carries ClpY derivatives in AD together with SulA or SuA(M89I) on BD. (A) LacZ expression. (B) Western blots. Strain notations correspond to those used in Fig. 2B.

rescence (excitation, 340 nm; emission, 460 nm). The ATPase activity was determined by the rate of decrease in the amount of NADH. The ATPase activity of the wild-type ClpY was used as a standard. Three independent experiments were performed with triplicate samples, and the SD was determined.

Statistical methods. The data from three experiments, each containing three replicates, were pooled together for statistical evaluation. Student's *t* test was applied for the comparison of two interested samples. A *P* value of <0.05 was considered statistically significant in this test.

RESULTS

Randomly mutagenized ClpY mutants, altered in the ATP-binding site, pore I, or pore II, associated with SulA and SulA(M89I). In the yeast two-hybrid system, ClpY interacts with its substrate SulA (26, 27). Yeast cotransformants carrying plasmids AD-*clpY* $^{+}$ and BD-*sulA*$^{+}$ [or *sulA*(*M89I*)] are capable of growing on Leu^{+}-selective media and are light blue on X-Gal plates because AD-ClpY and BD-SulA associate with each other, and the resulting complex activates dual reporter genes, resulting in *lacZ* gene expression and a Leu^{+} phenotype. Thus, through the yeast two-hybrid analysis and the bacterial physiological study, it was demonstrated earlier that the in-front double loops in domain I of ClpY are mostly involved in an initial recognition/tethering of SulA (27). In this study, additional ClpY mutants, ClpY(T87I), ClpY(G90D), and ClpY(G269C), were isolated with a mutation(s) in the ATP-binding site, pore I, or pore II, respectively (Fig. 1A and C).

To examine whether these ClpY mutants were still capable of association with SulA or SulA(M89I), the corresponding pB42AD derivatives were transformed into EGY48 carrying pGilda-BD-*sulA* or *sulA*(*M89I*). SulA(M89I) has activity similar to that of the wild-type SulA but retains a higher affinity toward ClpY (26). Yeast strains carrying AD-ClpY(T87I), AD-ClpY(G90D), or AD-ClpY(G269C) in combination with BD-SulA or SulA(M89I) produced colonies with β-galactosidase levels similar to those of yeasts carrying the wild-type ClpY; with ClpY(G90D), the level was slightly higher (Fig. 2A). In addition, the strains carrying these ClpY mutants grew on Leu^{+}-selective media. These results indicate that ClpY(T87I), ClpY(G90D), and ClpY(G269C) all bind to SulA or SulA(M89I). In addition, Western-blot analyses showed that ClpY and its derivatives were expressed well in yeasts (Fig. 2B).

Pore I mutant proteins ClpY(Y91F), ClpY(Y91S), and ClpY (V92F) interact with SulA. In previous biochemical analyses, whether ClpY pore I site mutants are capable of association with the natural substrates of ClpY was not clear (35). Therefore, the additional pore I mutations *clpY*(*Y91F*), *clpY*(*Y91S*), and *clpY*(*V92F*) were constructed by using two-step PCR. These three mutants were genetically identical to those previously isolated by Park et al. (35). Each mutant, in its side chain, was lacking either the hydroxyl group [ClpY(Y91F)] or the hydrophobic ring [ClpY(Y91S)] (Fig. 1C). ClpY(V92F), however, has a hydrophobic ring instead (Fig. 1C). Therefore, the mutated *clpY* gene was separately cloned into plasmid pB42AD, and the resulting plasmids pB42AD-*clpY*(*Y91F*), -*clpY*(*Y91S*), and -*clpY*(*V92F*) were each transformed into yeast strain EGY48 containing plasmid BD-*sulA* or BD-*sulA*(*M89I*). The transformants were tested on Leu^{+}-selective media, scored on X-Gal plates, and assayed for β-galactosidase activity. Yeast cells carrying ClpY(Y91F), ClpY(Y91S), or ClpY(V92F) with either SulA or SulA(M89I) grew on Leu^{+}-selective media and had β-galactosidase activities similar to those of cells carrying wild-type ClpY (Fig. 3A). Thus, ClpY(Y91F), ClpY(Y91S), and ClpY(V92F) all associate with SulA or SulA(M89I). In Western blot analyses, wild-type AD-ClpY and its derivatives were expressed well, except for AD-ClpY(Y91F) (Fig. 3B), which was expressed at a lower level but with a higher β-galactosidase activity; yet its control yeast also has a higher background level (Fig. 3A). Our results indicate that all of these

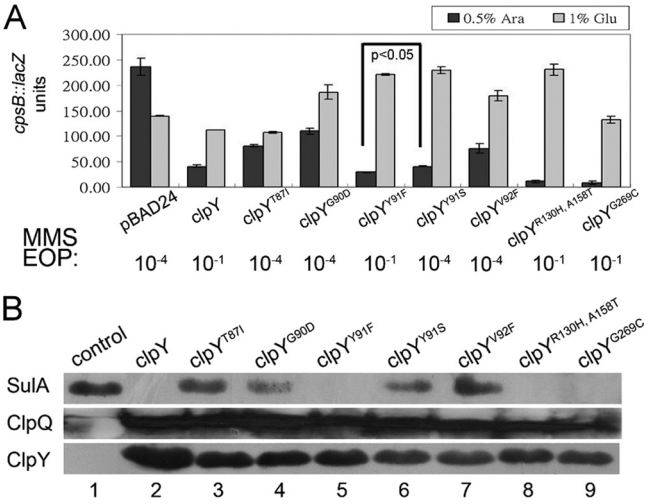

FIG. 4. Assays of *cpsB*::*lacZ* expression and MMS tests as well as Western blot analysis of *E. coli.* (A) β-Galactosidase activity of *cpsB*::*lacZ* fusions in the presence of 0.5% arabinose or 1% glucose and EOP values for each strain in MMS tests. $P < 0.05$ was considered statistically significant with Student's *t* test. Each strain has plasmids carrying wild-type ClpY or a ClpY mutant in the presence of a second plasmid carrying ClpQ. (B) Levels of HA-SulA, ClpQ, and ClpY derivatives present in each bacterial strain. Protein levels reflect amounts of protein remaining after proteolysis. Anti-HA monoclonal antibody was used to detect HA-SulA; anti-ClpQ and anti-ClpY polyclonal antibodies were used to detect ClpQ and ClpY. Samples were obtained from the indicated strains after growth to exponential phase in LB medium with triple antibiotics plus 0.5% arabinose and induction with 1 mM IPTG at 30°C for 3 h.

mutants, ClpY(Y91F), ClpY(Y91S), and ClpY(V92F), are still capable of association with SulA or SulA(M87I).

***In vivo*, a ClpY ATP-binding site mutant and certain ClpY pore I mutants do not degrade their substrates, as determined by the MMS test and *cpsB*::*lacZ* expression as well as Western blot analysis.** The biological activities of ClpY mutants and wild-type ClpY were subsequently examined in bacteria. The mutated *clpY* genes (their mutant derivatives are listed in Fig. 1C) were subcloned into pBAD24, and the resulting plasmids were designated as follows: ATP-binding site mutant, pBAD24-*clpY*(*T87I*); pore I mutants, pBAD24-*clpY*(*G90D*), -*clpY*(*Y91F*), -*clpY*(*Y91S*), and -*clpY*(*V92F*); pore II mutant, ClpY(G269C); and a newly isolated mutant, pBAD24-*clpY*(*R130H*, *A158T*), with mutations in the tip of domain I. These plasmids and the wild-type pBAD24-*clpY*$^+$ were transformed into AC3112 carrying pBAD33-*clpQ*$^+$. The cells, which expressed both ClpY and ClpQ, were resistant to MMS (EOP, about 10^{-1}) and expressed a lower β-galactosidase activity from the *cpsB*::*lacZ* fusion (Fig. 4A) due to the independent proteolysis of SulA and RcsA (22, 28). AC3112 cells that expressed ClpY(Y91F), ClpY(R130H, A158T), or ClpY(G269C) with ClpQ were also MMS resistant and produced lower β-galactosidase activities on the growth media containing arabinose (Fig. 4A, lanes 5, 8, and 9). In contrast, bacteria that expressed ClpY(T87I), ClpY(G90D), ClpY(Y91S), or ClpY(V92F) in the presence of ClpQ were sensitive to MMS (EOP $< 10^{-3}$) and produced higher β-galactosidase activities (Fig. 4A, lanes 3, 4, 6, and 7). However, ClpY(Y91F) has a positive effect, as does the wild-type ClpY (Fig. 4, lane 5). These results suggest that ClpY(T87I), with a mutation in the ATP-binding site, and ClpY(G90D), ClpY(Y91S), and ClpY(V92F), each with a pore I site mutation, are defective in degradation of their natural substrates; these observations are consistent with those of previously reported biochemical studies (35).

Levels of SulA, ClpQ, and ClpY in each bacterial strain were also assayed by Western blot analysis. As in the previous study, strain AC3112(pTH18kr-*ha-sulA*$^+$) cells that carried pBAD33-*clpQ*$^+$ together with pBAD24-*clpY*$^+$ or its derivatives were constructed. The corresponding bacterial strains, in LB medium with the appropriate antibiotics plus 0.5% arabinose, were grown to log phase and induced with IPTG. After 3 h, cell extracts were analyzed on Western blots using anti-HA, anti-ClpQ, and anti-ClpY antibodies. The control strain with pTH18kr-*ha-sulA*$^+$, pBAD33, and pBAD24 was used for detection of HA-SulA in the absence of ClpQ and ClpY. An accumulation of SulA was observed in bacteria that expressed ClpY(T87I), ClpY(G90D), ClpY(Y91S), or ClpY(V92F) in the presence of ClpQ (Fig. 4B, lanes 3, 4, 6, and 7). However, ClpY(Y91F), ClpY(R130H, A158T), and ClpY(G269C) were all capable of degrading SulA (Fig. 4B, lanes 5, 8, and 9). ClpQ and ClpY as well as its derivatives are expressed well in all cases, although levels of the mutant proteins were somewhat reduced relative to those of the wild-type ClpY (Fig. 4B, lanes 2 to 9).

ClpY pore II mutant proteins, ClpY(E266Q), ClpY(S267L), and ClpY(S268L), interact with SulA(M89I). ClpY pore II site mutants, *clpY*(*E266Q*), *clpY*(*S267L*), and *clpY*(*S268L*), were also constructed by mutagenesis using two-step PCR. Again, each mutant in its side chain was changed to a polar residue with E266Q or carrying the hydrophobic group with S267L and S268L. The three *clpY* mutant genes were cloned into pB42AD, and the resulting plasmids pB42AD-*clpY*(*E266Q*), -*clpY*(*S267L*), and -*clpY*(*S268L*) were transformed into the yeast coexpressing BD-*sulA* or BD-*sulA*(*M89I*), and the transformants were tested for the Leu$^+$ phenotype and β-galactosidase activity. ClpY(E266Q), ClpY(S267L), and ClpY(S268L) all interacted with SulA(M89I), producing cell growth in the absence of leucine and increased β-galactosidase levels (Fig. 5A). Western blot analysis showed that all three of these mutant proteins, ClpY(E266Q), ClpY(S267L), and ClpY(S268L), were expressed as well as the wild-type ClpY (Fig. 5B). pBAD24-*clpY*(*E266Q*), -*clpY*(*S267L*), and -*clpY*(*S268L*) were constructed, and all of them were transformed into AC3112 with pBAD33-*clpQ*$^+$. In bacteria, ClpY(E266Q), ClpY(S267L), and ClpY(S268L) pore II mutants as well as wild-type ClpY were capable of degrading SulA and RcsA, since bacteria with any of these mutant proteins were MMS resistant and had lower β-galactosidase activities (Fig. 5C); in addition, there was no accumulation of SulA for either of the mutants in bacteria carrying pTH18kr-*ha-sulA*$^+$ (Fig. 5D).

ClpY(Y408A), with a mutation in domain C, associated with SulA without efficiently degrading it. A ClpX mutant with a single amino acid substitution in its domain C at position 385 (tyrosine-to-alanine substitution) binds to its substrate but lacks unfolding activity (13). Yet the real mechanism behind the observation is not clear. Based on the amino acid sequence alignment of the C domains of ClpY and ClpX, the corresponding mutation in *clpY*, *clpY*(*Y408A*), was introduced by site-specific mutagenesis and the mutated gene was cloned into

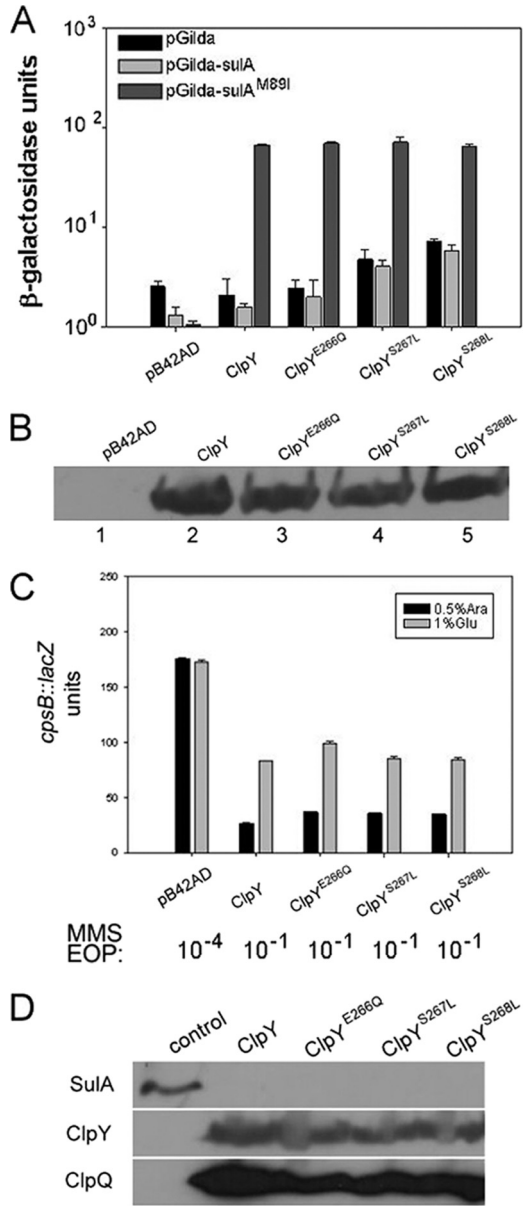

FIG. 5. Effect of the ClpY-pore II mutations on association of ClpY with SulA or SulA(M89I). (A) Assays of β-galactosidase in yeast. (B) Western blots of ClpY and its mutant derivatives. (C) *cpsB*::*lacZ* expression and MMS tests for each bacterial strain. (D) Western blot analysis of SulA, ClpQ, ClpY, and ClpY(E266Q), ClpY(S267L) or ClpY(S268L) after proteolysis. Notation and methods used in panels A to D are similar to those used for Fig. 2A and B and Fig. 4A and B, respectively.

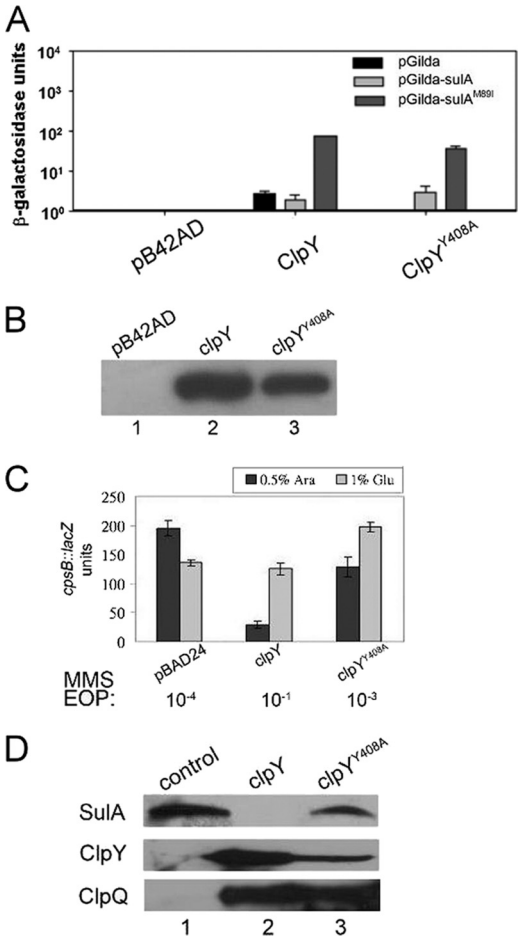

FIG. 6. Effects of the ClpY(Y408A) mutation on association with SulA or SulA(M89I). (A) β-Galactosidase activity in yeast. (B) Western blots of ClpY and ClpY(Y408A) in the AD fusion protein using an anti-HA antibody. (C) *cpsB*::*lacZ* expression and MMS tests for each bacterial strain. (D) Western-blot analysis of the residual SulA, ClpQ, ClpY, and ClpY(Y408A) remaining after proteolysis. Strain notations and conditions used for parts A to D are similar to those indicated in Fig. 5.

pB42AD. Accordingly, Y408A has removed all of the side chain in the 408th residue. Plasmid pB42AD-*clpY*(*Y408A*) was introduced into yeasts that carry pGilda-*sulA*$^+$ or pGilda-*sulA*(*M89I*). The resultant transformants were able to grow on Leu$^+$-selective media and had higher β-galactosidase activities (Fig. 6A). Western blot analysis has shown that ClpY(Y408A) was expressed at a lower level (Fig. 6B). These results indicate that ClpY(Y408A), carrying a mutation in domain C, is still capable of associating with its natural substrates.

The biological activity of ClpY(Y408A) was then measured with the plasmids pBAD24-*clpY*(*Y408A*) and pBAD33-*clpQ*$^+$, which were cotransformed into the bacterial strain AC3112. Notably, the resulting transformants produced higher β-galactosidase activities and were more sensitive to MMS than wild-type transformants, following arabinose induction (Fig. 6C). These results indicate that the ClpY(Y408A) proteolytic complex is defective in targeting its natural substrates. Furthermore, in the presence of ClpQ, SulA accumulated in the bacterial strain carrying ClpY(Y408A), in contrast to cells expressing wild-type ClpY. However, ClpY(Y408A) itself was expressed at a lower level than wild-type ClpY (Fig. 6D), whereas an equal amount of ClpQ was expressed in the various bacteria. These results indicate that ClpY(Y408A) led to an accumulation of SulA in the presence of ClpQ, most probably due to its own unstable conformation.

***In vitro* protein pulldown analyses of ClpY and its derivative mutants with MBP-SulA.** The *in vitro* pulldown analyses were also used to analyze ClpY and its derivative mutants listed

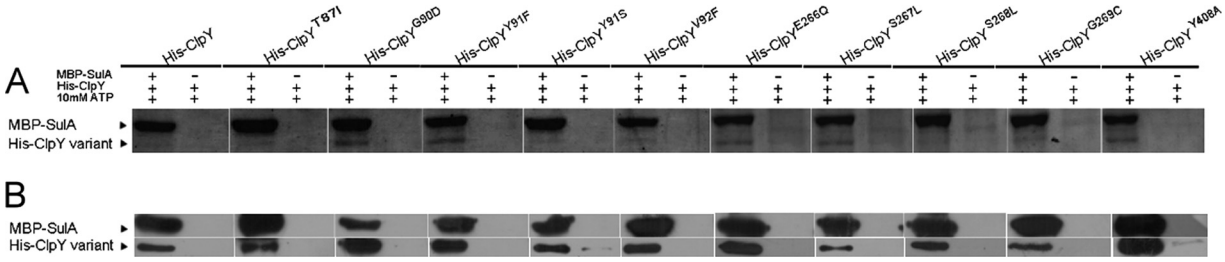

FIG. 7. Pulldown analyses. The wild-type ClpY and its derivative mutant proteins with His tags were used for precipitation with MBP-SulA in the presence of ATP. Each lane is as indicated. Equal amounts of ClpY and its derivatives were normalized by using the Bradford protein assays. The top panel shows the results from precipitated samples running on an SDS-PAGE gel and stained with Coomassie blue R-250. The bottom panel shows results from sample detection in Western blot analyses by using the anti-MalE and anti-ClpY multiserum.

above for their association with MBP-SulA. ClpY and its above derivative mutants were each purified using His tag affinity purification methods, whereas MBP-SulA was purified using amylose resin (NEB) methods and the concentration of each protein was determined (see Materials and Methods). Each ClpY mutant protein and the wild-type ClpY were equivalently tested for their own pulling down with MBP-SulA in the presence of ATP. As shown in Fig. 7, each of the various ClpY mutants was capable of pulling down with MBP-SulA, seen in the SDS-PAGE gel and in the Western blot analyses. An increased amount of the ClpY(G90D), ClpY(Y91F), ClpY(E266Q), or ClpY(Y408A) mutant for its association with MBP-SulA was observed in both detections. Therefore, these results coincided with the observations in the two-hybrid analyses; yet both assays showed subtle but visible association. It was also indicated that certain ClpY mutants, with a mutation in the pore I site or the pore II site, are capable of fastening their natural substrates.

***In vitro* analysis of purified ClpY(T87I), ClpY(Y408A), and ClpY for proteolytic activity, self-oligomerization, and association with ClpQ.** ClpY, and its derivative ClpY(T87I) and ClpY(Y408A) mutants were then *in vitro* tested for the ability to degrade MBP-SulA in the presence of ClpQ. Also, using the Image J program, the relative levels of remaining substrates were determined for the degradation assays. As shown in Fig. 8A, with ClpQ, ClpY degraded 63% ± 2% of the MBP-SulA in 4 h (Fig. 8A, far left), but there was 11% ± 1% degradation of MBP-SulA by ClpY(T87I) (Fig. 8A, second panel); ClpY(Y408A) degraded at most 44% ± 1% of the MBP-SulA in the same time period (Fig. 8A, third panel). The remaining percentage of MBP-SulA with ClpY or its derivatives was additionally plotted (Fig. 8A, far right).

In addition, a cross-linking analysis showed that ClpY(T87I) itself formed an oligomer in the presence or absence of ATP-γS (Fig. 8B, middle, lanes 5 and 6). However, its hexamer was not capable of forming a higher-order oligomer with ClpQ (Fig. 8B, lanes 7 and 8). In contrast, the wild-type ClpY hexamer did form an oligomer with ClpQ (Fig. 8B, middle, contrast lane 4 with lanes 1, 2, and 3). Each ClpQ and ClpY alone was shown separately as the negative controls (Fig. 8B, far left). At a normal concentration, ClpY(Y408A) was barely observed to form an oligomer in the presence of ATP-γS (Fig. 8B, middle, compare lane 10 with lanes 2 and 9, without ATP-γS). As shown, in the presence of ATP-γS, ClpY(Y408A) formed the oligomers with ClpQ but much less than the wild-type ClpY (Fig. 8B, middle, compare lane 12 with lane 4 and lane 11, without ATP-γS). In the presence of ADP, both ClpY and ClpY(T87I) hardly formed a complex with ClpQ (Fig. 8C). Thus, the ClpY(Y408A) hexamer by itself was not stable *in vitro*; this is likely correlated to the *in vivo* observation described above for an instable ClpY(Y408A) molecule.

Thermodynamic analysis of ATP-γS-binding and ATPase activity. Since ClpY(T87I) carries a mutation in its ATP-binding site, by the isothermal titration calorimetry (ITC) method, the ATP binding was separately analyzed for ClpY(T87I) and wild-type ClpY. Each protein molecule was titrated with ATP-γS. The raw data were fitted to the one-set site binding model (Fig. 9A). Both proteins were capable of association with three or four ATP-γS molecules, and these results are consistent with those of the earlier reports for the association of ClpY with ATP (43). However, it was difficult to determine the K_d values in both ITC experiments (data not shown). The ATPase activity was subsequently measured for ClpY(T87I) and wild-type ClpY. In the presence of MBP-SulA and ClpQ, ClpY(T87I) has about one-fourth of the ATPase activity of wild-type ClpY (Fig. 9B). Thus, ClpY(T87I) in its hexameric form was defective in ATPase activity.

DISCUSSION

In yeast two-hybrid assays, ClpY associated with its natural substrates SulA or SulA(M89I) (27). Similarly, ClpY pore I mutants, pore II mutants, and the domain C mutant ClpY(Y408A) all associated with SulA and/or SulA(M89I). However, in bacterial cells in the presence of ClpQ, certain ClpY mutants [ClpY(T87I), ClpY(G90D), ClpY(Y91S), and ClpY(V92F)] were not capable of degrading SulA to the same extent as the wild-type ClpY. Moreover, β-galactosidase expression from *cpsB*::*lacZ* in bacteria was higher for these mutants due to reduced degradation of RcsA. In contrast to the results obtained with ClpY(T87I) and the pore I mutants, in the presence of ClpQ, the pore II mutants ClpY(E266Q), ClpY(S267L), ClpY(S268L), and ClpY(G269C) were capable of degrading SulA. In addition, ClpY(Y408A), with a mutation in domain C, was not capable of degrading the substrates. These results indicate that these ClpY mutants altered in pore I, pore II, or domain C have no negative effect on the binding of a natural substrate, but mutations in the ATP-binding site, pore I, and domain C do affect subsequent proteolysis of the substrates.

ClpY recognizes and binds to fluorescence-labeled P22 Arc pro-

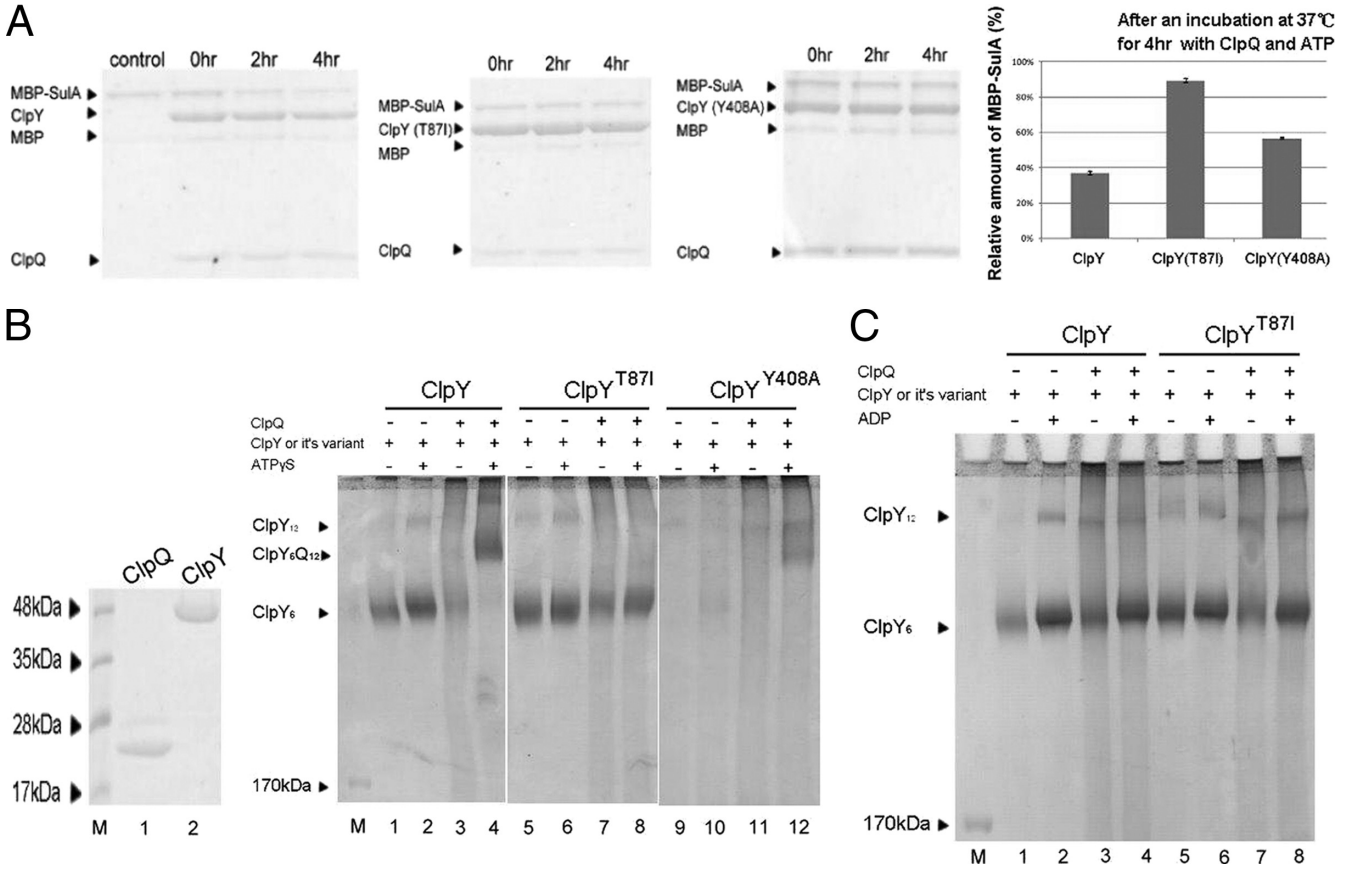

FIG. 8. Analysis of the biological activity of ClpY, ClpY(T87I), and ClpY(Y408A) *in vitro*. (A) MBP-SulA degradation by ClpY, ClpY(T87I), and ClpY(Y408A) in the presence of ClpQ. ClpY or its derivatives (about 4.05 μM) were incubated with ClpQ (6.62 μM), MBP-SulA (1.6 μM), and ATP (5 mM) at 37°C for the indicated amounts of time. The samples were then subjected to SDS-PAGE on a 12.5% gel, followed by staining with Coomassie blue R-250. The analysis of MBP-SulA decay by ClpY or its derivatives was plotted (right). The remaining percentage of MBP-SulA for degradation and the standard deviations were calculated from three independent experiments. (B) Cross-linking analysis of ClpY(T87I), an ATPase mutant, as well as ClpY(Y408A) and wild-type ClpY. (Left) Lane M, molecular mass marker; lane 1, ClpQ; lane 2, ClpY (without the cross-linker). (Middle) Lane M, molecular mass marker; lane 1, ClpY; lane 2, ClpY with ATP-γS; lane 3, ClpY and ClpQ; lane 4, ClpY and ClpQ with ATP-γS; lane 5, ClpY(T87I) without ATP-γS; lane 6, ClpY(T87I) with ATP-γS; lane 7, ClpY(T87I) and ClpQ without ATP-γS; lane 8, ClpY(T87I) and ClpQ with ATP-γS; lane 9, ClpY(Y408A) without ATP-γS; lane 10, ClpY(Y408A) with ATP-γS; lane 11, ClpY(Y408A) and ClpQ without ATP-γS; lane 12, ClpY(Y408A) and ClpQ with ATP-γS. (Lanes 1 through 12 include the cross-linker.) (Right) Analysis of ClpY and ClpY(T87) cross-linked with ClpQ in the presence of ADP. The reaction mixtures containing ClpY or ClpY(T87I) (4 μM; 6-mer) and 1 mM ATP-γS or 1 mM ADP were preincubated with ClpQ (4 μM; 12-mer) for 5 min at 37°C; the mixture was then combined with 0.4% (vol/vol) glutaraldehyde to perform the cross-linking reaction during a 20-min incubation at 37°C. The samples were then subjected to SDS-PAGE on a 4 to 12% gradient gel, followed by staining with Coomassie blue R-250. Lane M, molecular mass marker; lane 1, ClpY without ADP; lane 2, ClpY with ADP; lane 3, ClpY and ClpQ without ADP; lane 4, ClpY and ClpQ with ADP; lane 5, ClpY(T87I) without ADP; lane 6, ClpY(T87I) with ADP; lane 7, ClpY(T87I) and ClpQ without ADP; lane 8, ClpY(T87I) and ClpQ with ADP.

tein (an artificial substrate) in the presence of ATP-γS and Mg^{2+} (5). ATP-bound ClpY was capable of recognizing the natural substrates as part of the ClpYQ complex. All the ClpY mutants described above and ClpY itself with the bound ATP(s) were capable of associating with MBP-SulA. Specifically, ClpY(Y91F) or ClpY(E266Q) showed a marked association with the substrates. These results suggest that the pore I or pore II site of ClpY is capable of strengthening the binding affinity of substrates.

However, ClpY(T87I) itself forms a hexameric ring in the presence or absence of ATP-γS. Yet its ATP-γS hexamer ring was not capable of forming a complex with ClpQ in cross-linking analyses (Fig. 8). These results explain why ClpY(T87I) was not capable of degrading its natural substrates in the presence of ClpQ. Previously, it was proposed that the C-terminal tail of ClpY becomes available for interaction with ClpQ when an ATP-dependent conformational change occurs in ClpY (39, 42). It was also reported that hexamers of ClpY and ClpQ associate with each other in the presence of 1 μM [ATP] (28). Interestingly, ClpY(T87I) itself forms a hexameric ring in the absence of ATP-γS, yet its hexamer is still capable of binding with ATP-γS in the ITC analysis. However, ClpY(T87I) has a binding affinity a little lower than that of the wild-type ClpY, since it has a slightly less ΔH value in titration by ATP-γS in an ITC analysis. Additionally, the cross-linking analyses demonstrated that, in the presence of ATP-γS, the newly formed ClpY(T87I) hexamer was less accumulated than that of the wild-type ClpY. Taken together, dissimilar to results with the previously isolated ATP-binding site defective mutants (32, 40), our results directly support that ClpY(T87I) is lacking a conformational change for association with ClpQ.

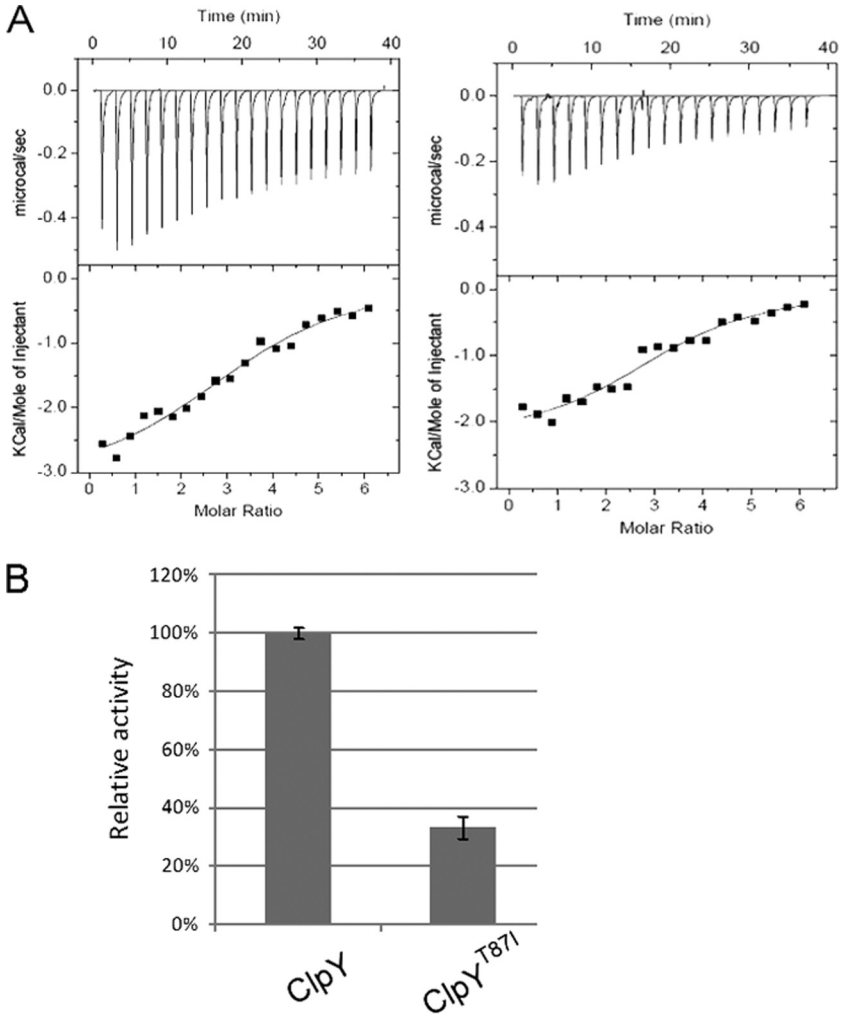

FIG. 9. The relative ATPase activity and the ATP binding analysis. A nonlinear regression fit of these data was used to determine the change in enthalpy (ΔH) and the change in entropy (ΔS), and the free energy change (ΔG) was calculated. The binding of ATP-γS to the ClpY or ClpY(T87I) in buffer was exothermic. (A) For ClpY, the binding ratio (ATP-γS to ClpY) (N) = 3.5 ± 0.1, $\Delta H = -3.2 \pm 0.2$ kcal/mol, and $\Delta G = -8.2$ kcal/mol (left); for ClpY(T87I), $N = 3.2 \pm 0.2$, $\Delta H = -2.3 \pm 0.2$ kcal/mol, and $\Delta G = -8.7$ kcal/mol (right). (B) ClpY or the mutant derivatives, together with ClpQ and MBP-SulA, were incubated at 37°C for 2 h and then the ATPase reaction mix was added. The decrease in the amount of ATP was measured fluorometrically. The decrease in ATP in the presence of wild-type ClpY with ClpQ is defined as 100%.

The glycine at position 90 of ClpY was proposed to provide pore I region flexibility for transferring of the natural substrate into the inner core of ClpQ, yet its mutant derivatives cannot form a complex with ClpQ (35). In our study, ClpY(G90D) binds SulA(SulAM89I) more efficiently than wild-type ClpY in both *in vivo* and *in vitro* assays. This also could be an indirect effect since in the X-ray crystallography, three conformations of ClpY were observed due to differences in the relative orientations of αβ and the α subdomain in different nucleotide states (45); these could also place domain I in a direction that allows fine-tuning in substrate binding at the pore site (30, 45). Since the ClpY(G90D) mutant is defective in the degradation of its substrates (Fig. 4), it is also likely that it possesses a lower affinity for complex formation with ClpQ.

The other two ClpY pore I mutants, ClpY(Y91S) and ClpY(V92F), were defective *in vivo* in targeting natural substrates when ClpQ was present (Fig. 5). In contrast, ClpY(Y91F) was capable of degrading substrates in the presence of ClpQ. ClpY(Y91F) has a hydrophobic ring at position 91, but ClpY(Y91S) does not. Since ClpY(Y91S) maintains SulA-binding activity in our assay, this mutant is defective in translocation of the substrates. Therefore, the results also support that a hydrophobic group at position 91 of ClpY is necessary to shift substrates into the inner core of ClpQ for degradation. It has been suggested before that a valine at position 92 is required for the binding of specific substrates to the pore I site of the ClpYQ complex (19, 35). We found that ClpY(V92F) interacted with SulA in both *in vivo* and *in vitro* analyses. These results are consistent with the analysis of another ATP-dependent protease, ClpA, for which a deletion of the GYVG pore site has no effect on substrate binding (11, 30). The substrates are still binding in the mutant ClpY complex containing the pore I site mutation(s).

The pore II mutants ClpY(E266Q), ClpY(S267L), ClpY (S268L), and ClpY(G269C) actively associated with SulA. In the degradation of SulA, their bacterial cells all expressed

MMS resistance, and no residual SulA was observed in Western blot analysis (Fig. 5 and 7). These results suggested that the pore II site is not as active as the pore I site for the translocation of the substrates.

In contrast, ClpY(Y408A) binds well to SulA but appears to be defective in degrading it when ClpQ is present. The purified ClpY(Y408A) is slightly self-oligomerized in the presence of ATP-γS. Also, in bacterial cells, ClpY(Y408A) was detected at much lower levels than wild-type ClpY. Most probably, the oligomeric form of ClpY facilitates its *in vivo* subunit stability; ClpY(Y408A) is lacking the side chain, at the 408th position, and is not capable of a flexible association with the neighboring subunit, therefore promoting oligomer instability. Yet with ATP-γS, in the presence of ClpQ, ClpY(Y408A) forms a subtle protease complex [$ClpY(Y408A)_6ClpQ_{12}$] with ClpQ as well as itself [$ClpY(Y408A)_{12}$]; these alterations suggest that ClpQ is stabilizing the oligomeric form of ClpY(Y408A), directly or indirectly. In addition, in the pulldown assays, ClpY(Y408A), in the presence of the larger amount of ATP, showed higher binding affinity toward MBP-SulA, and the reason is unknown, although it is likely due to an indirect effect.

From our study, a tip mutant ClpY(R130H, A158T) also binds substrate normally (data not shown). However, our results indicated that the pore I or pore II site of ClpY has a role in binding of the substrates; i.e., the ClpY(Y91F) mutant or ClpY(E266Q) has enhanced binding activity. Our study indicated that the substrates were positioned to a site adjoining the central pore in the ClpY hexamer prior to translocation for the final degradation by ClpQ. Since all of the pore I or pore II site ClpY mutants are capable of binding with the substrates, regardless of whether the proteolysis occurs or not, our results suggest that an unfolding/translocation of the substrates at the pore site in ClpYQ complex is a determinant for the final degradation of the substrates. In addition, it was shown before that domain I is necessary for the degradation of the substrates (23, 27). Two receptor sites, the tethering site (T) and the pore site (P), for substrate binding were brought out in the ClpY structure (27, 43). Also, the computational simulations indicated that the low-native-content substrate protein conformations out of an unfolding are stabilized by the nearby region in domain I of ClpY (20). In future studies, it will be interesting to determine how the unfolding/translocation is temporally processed at the pore site with the subsidiary of domain I for the targeted substrates, thereby completing the degradation process by the ClpYQ complex in coupling with the ATPase activity, which affects conformational changes upon ATP binding and hydrolysis.

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