Analysis of the Cooperative ATPase Cycle of the AAA+ Chaperone ClpB from *Thermus thermophilus* by Using Ordered Heterohexamers with an Alternating Subunit Arrangement*

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**Background:** Ring-shaped ClpB hexamer hydrolyzes ATP and reactivates protein aggregates.

**Results:** Intercalation of ATPase defective subunits into the hexamer every other subunit hampered its ATPase and disaggregation activities.

**Conclusion:** ClpB cooperatively hydrolyzes ATP, and this cooperativity is crucial for protein disaggregation.

**Significance:** This study presents a new method to study the cooperativity of homo-oligomeric proteins and provides insights into the common mechanism of AAA+ ATPases.

The ClpB/Hsp104 chaperone solubilizes and reactivates protein aggregates in cooperation with DnaK/Hsp70 and its cofactors. The ClpB/Hsp104 protomer has two AAA+ modules, AAA-1 and AAA-2, and forms a homohexamer. In the hexamer, these modules form a two-tiered ring in which each tier consists of homotypic AAA+ modules. By ATP binding and its hydrolysis at these AAA+ modules, ClpB/Hsp104 exerts the mechanical power required for protein disaggregation. Although ATPase cycle of this chaperone has been studied by several groups, an integrated understanding of this cycle has not been obtained because of the complexity of the mechanism and differences between species. To improve our understanding of the ATPase cycle, we prepared many ordered heterohexamers of ClpB from *Thermus thermophilus*, in which two subunits having different mutations were cross-linked to each other and arranged alternately and measured their nucleotide binding, ATP hydrolysis, and disaggregation abilities. The results indicated that the ATPase cycle of ClpB proceeded as follows: (i) the 12 AAA+ modules randomly bound ATP, (ii) the binding of four or more ATP to one AAA+ ring was sensed by a conserved Arg residue and converted another AAA+ ring into the ATPase-active form, and (iii) ATP hydrolysis occurred cooperatively in each ring. We also found that cooperative ATP hydrolysis in at least one ring was needed for the disaggregation activity of ClpB.

Bacterial ClpB and its yeast homolog Hsp104 contribute to the thermotolerance of cells (1, 2). ClpB/Hsp104 reactivates protein aggregates in concert with the DnaK/Hsp70 chaperone system by utilizing ATP hydrolysis (3–9). ClpB/Hsp104 is a member of the Clp/Hsp100 family, a subfamily of the ATPases associated with diverse cellular activities (AAA+ proteins) superfamily (10, 11). AAA+ proteins are associated with various cellular activities, including membrane fusion, DNA replication, and protein remodeling, and share the AAA+ module (ATPase domain), which consists of large and small subdomains (12). The large subdomain contains the Walker A motif (GAXGXGKT, where X is any amino acid), the Walker B motif (hhhhDE, where h is a hydrophobic residue), and the conserved arginine residue (called the Arg finger). The Walker A and Walker B motifs are critical for ATP binding and hydrolysis, respectively (13). The Arg finger is necessary for ATP hydrolysis and intersubunit communication (13). Members of the AAA+ family are divided into two types, type I and type II, possessing one and two AAA+ module(s) in a polypeptide, respectively. ClpB is a type II AAA+ protein and consists of an N-terminal domain, two AAA+ modules (AAA-1 and AAA-2), and a middle domain (14). ClpB forms a two-tiered ring-shaped hexamer in a process dependent on salt concentration, protein concentration, temperature, and nucleotide binding conditions (15–21). Structural changes in the ClpB hexamer, coupled with ATP binding and hydrolysis, generate the mechanical power required for protein disaggregation. Because a ClpB hexamer contains 12 ATPase domains, the ATPase cycle and chemomechanical coupling are thought to be complex and highly coordinated.

Although several groups have studied how the ATPase cycle of ClpB/Hsp104 proceeds and couples with disaggregation, the details are still unclear because of its complexity and differences between species (22–29). In some of these studies, experiments using heterohexamers consisting of two types of ClpB subunits were performed (24–26, 28, 29). However, because the heterohexamers used in these experiments were prepared by simple mixing of the ClpB mutants, they were not uniform; the arrangement and the number of each type of subunit in the hexamers varied. Polymorphisms of the heterohexamers limited the interpretation of these data.

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To resolve this problem, we employed a new method to control the order of subunits in the hexamer in the current study. We introduced cysteine substitutions at the subunit interface to form a disulfide bond between adjacent subunits. Cross-linked dimers of ClpB were successfully prepared by forming disulfide bonds on the subunit interfaces in the AAA-1 ring or the AAA-2 ring. The heterohexamer that consisted of three pairs of cross-linked dimers was uniform, and within this structure, two cysteine mutants were arranged alternately. We combined these cysteine mutants with additional mutations to prepare heterohexamers and examined their biochemical properties. We found that each AAA+ module independently bound ATP and cooperatively hydrolyzed them in each ring. We also found that cooperative ATP hydrolysis was crucial for effective disaggregation.

EXPERIMENTAL PROCEDURES

Plasmids—The recombinant plasmid pMCB1 containing a ClpB gene from Thermus thermophilus was used for a mutagenesis template (5). Site-directed mutagenesis was performed by overlap extension PCR method using Ex Taq DNA polymerase (30, 31). The mutations were confirmed by DNA sequence analysis.

Proteins—α-Glucosidase from Bacillus stearothermophilus and rabbit lactate dehydrogenase were purchased from Sigma. Rabbit pyruvate kinase was purchased from Roche. 3-Deoxy-isopropylmalate dehydrogenase (IPMDH)2 from T. thermophilus was expressed in Escherichia coli BL21 (DE3) and purified as described (32). ClpB from T. thermophilus (TClpB) and its mutants were expressed in E. coli BL21 (DE3) and purified as described previously (33), except that mutants having cysteine residue were purified in the presence of 1 mM diithiothreitol. DnaK, DnaJ, and GrpE from T. thermophilus (TDnak, TDnaj, and TGGrpE, respectively) were expressed in E. coli BL21 (DE3) carrying pMDK6 (TDnak), pMDJ10 (TDnaj), and pMGE3 (TGGrpE), respectively, and purified as described previously (34–37). Concentrations of substrate proteins were expressed as monomers, and those of T. thermophilus chaperones are noted as the monomers for TDnak and TDnaj, dimers for TGGrpE, monomers, or hexamers as indicated for TClpB and its mutants.

Preparation of TClpB Cross-linked Dimers—Two types of cysteine mutants of TClpB were mixed (1–10 μM hexamer) and oxidized by incubation in 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, and 50 μM CuCl2 in the presence or absence of 3 mM ATP at 55 °C for 60 min. The CuCl2 was removed on a disposable gel filtration column. Oxidized TClpB mutants were analyzed with SDS-PAGE on a 6% polyacrylamide gel in the absence of 2-mercaptoethanol. The bands of the cross-linked dimer and monomer TClpB were visualized with Coomassie Brilliant Blue and quantified using a Molecular Imager FX Pro Plus (Bio-Rad) multi-imager system. The cross-linking efficiency was then calculated. When the cross-linking efficiency was less than 90%, further purification was performed with a TSK G-3000SWXL HPLC gel filtration column (Tosoh, Tokyo, Japan) equilibrated with 50 mM MOPS-NaOH (pH 7.5), 500 mM KCl, 5 mM MgCl2, and 2 mM ATP. The cross-linking efficiencies of all TClpB dimers were about equal to or more than 90% (data not shown).

Gel Filtration Analysis—Gel filtration analyses of TClpB mutants were performed as described previously (38) using a TSK G-3000SWXL HPLC gel filtration column (Tosoh). One hundred microliters of purified TClpB mutants (1.73 μM hexamer) was applied to a gel filtration column equilibrated with 50 mM MOPS-NaOH (pH 7.5), 5 mM MgCl2, 2 mM ATP, and the indicated concentrations of KCl at a flow rate of 0.5 ml/min at 55 °C. Absorbance was monitored at 290 nm.

Measurement of Nucleotide Binding—The fluorescent nucleotide analog 2′(3′)-O-′-methylaniolyl-aminoadenosine-5′-diphosphate (Mant-ADP) was purchased from Life Technologies. Mant-ADP binding to TClpB was monitored by determining the increase in fluorescence of Mant-ADP, as described previously (20). The displacement of Mant-ADP to ADP or ATP was monitored by determining the decrease in fluorescence of Mant-ADP, as described previously (20). All measurements were performed with a FP-6500 fluorometer (Jasco, Tokyo, Japan) at 55 °C. The apparent dissociation constants of Mant-ADP, ADP, and ATP for TClpB monomer were calculated by fitting the data as described previously (20), except that the concentrations of TClpB monomer having the ability to bind nucleotide were used for the fitting. The data were analyzed by KaleidaGraph 4.1 (Synergy Software, Reading, PA).

Measurement of ATPase Activity—The ATPase activities of wild-type or mutant TClpB were measured spectrophotometrically with an ATP-regenerating system at 55 °C. The assay mixture contained 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, 0.2 mM nicotinamide adenine dinucleotide, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, 2.5 mM phosphoenolpyruvic acid, 3 mM ATP, and 5 mM tris-(2-carboxyethyl) phosphine hydrochloride when indicated. The reaction was initiated by the addition of 0.05 μM wild-type or mutant TClpB hexamers as indicated, and the changes in absorbance at 340 nm were monitored using a V-650 spectrophotometer (Jasco).

Reactivation of Heat-aggregated Proteins—α-Glucosidase and IPMDH were used as substrate proteins. Substrate proteins (0.2 μM monomer) in a mixture containing 50 mM MOPS-NaOH (pH 7.5), 150 mM KCl, 5 mM MgCl2, 3 mM ATP, and 5 mM tris-(2-carboxyethyl) phosphine hydrochloride when indicated were heat aggregated by incubation at 73 °C for 10 min (α-glucosidase) or 89 °C for 30 min (IPMDH). Subsequently, chaperones (final concentration of 0.6 μM TDnak, 0.2 μM TDnaj, 0.1 μM TGGrpE, and 0.05 μM wild-type or mutant TClpB hexamers as indicated) were added to the reaction mixture, and the mixture was incubated at 55 °C for 90 min. Recovered enzymatic activity was assayed as described previously (32).

RESULTS

Inter-subunit Disulfide Cross-linking between Adjacent Subunits of TClpB—We designed single cysteine mutants that could potentially form disulfide bonds between adjacent subunits. According to the high resolution hexameric structures of other Clp/Hsp100 proteins, HsU and ClpC, the small subdomain of the AAA+ module interacted with the large subdomain of the AAA+ module of the adjacent subunit (39, 40).

2 The abbreviation used is: IPMDH, 3-deoxy-isopropylmalate dehydrogenase.
Therefore, we focused on the interface to select candidate amino acids to be replaced with cysteine (Fig. 1). On the AAA-1 module, we selected Gln184 and Arg189, which were present on the outer face of the large subdomain, and Tyr348, His351, and Ala390, which were present on the inner face of the small subdomain. On the AAA-2 module, we selected Arg568, Asp572, Arg576, and Leu581 from the outer face of the large subdomain and Arg776, Glu779, Ala821, and Gln822 from the inner face of the small subdomain. Single mutants of cysteine were mixed in various combinations and oxidized by incubation with CuCl2 in the absence or presence of ATP. The efficiency of the intersubunit disulfide bond formation was estimated by nonreducing SDS-PAGE (Fig. 2). Among all the tested combinations, one or two upshifted bands were observed. Because these mutants had only one cysteine (wild-type TClpB had no intrinsic cysteine), the band should correspond to the cross-linked homodimer or heterodimer. Thus, upon incubating Q184C and A390C without ATP, the majority of TClpB appeared as an upshifted band (Fig. 2A). Because the band did not appear following separate incubation of Q184C or A390C, this observation could only be due to the heterodimer. Among the combinations of various AAA-1 mutants, Q184C and A390C showed the highest yield (86%) of cross-linked heterodimers. However, for different combinations of AAA-2 mutants, the highest yield (94%) was observed for the cross-linked heterodimer of R576C and A821C incubated with 3 mM ATP (Fig. 2, B–D). Henceforth, these cross-linked heterodimers are referred to as D1 dimer and D2 dimer, respectively.

Biochemical Properties of D1 and D2 Dimers—A stable hexamer was eluted by the HPLC size exclusion chromatography of wild-type TClpB at 55 °C in the presence of 2 mM ATP and 150 mM KCl. However, increasing the concentration of KCl led to the destabilization of the hexameric structure, and the elution peaks were delayed (Fig. 3). Both D1 and D2 dimers could form stable hexamers under the same conditions, and the high concentration of KCl caused little destabilization.

In the presence (reducing conditions) and absence (nonreducing conditions) of tris-(2-carboxyethyl) phosphine hydrochloride, wild-type TClpB hydrolyzed ATP at rates of 49 and 46 min⁻¹, respectively. Under reducing conditions, the ATPase rates of D1 and D2 dimers were 50 and 17 min⁻¹, respectively, whereas those under nonreducing conditions were 36 and 27 min⁻¹, respectively. Under reducing conditions, the ATPase rate of the D2 dimer was relatively low (35% of that of the wild-type protein). This result may have been caused by the disturbance of the subunit interface caused by the cysteine substitutions. However, the defect was partially compensated by forming disulfide bond. Such compensation was also found in a similar experiment performed using ClpX, the other Clp/Hsp100 protein (41).

Next, we tested the disaggregation activities of wild-type and cross-linked TClpB in cooperation with 7DnaK system. α-Glucosidase was heat-aggregated by incubation at 73 °C for 10 min. Under reducing and nonreducing conditions, wild-type TClpB could reactivate 46 and 34% of the aggregated α-glucosidase, respectively. Under reducing conditions, the D1 and D2 dimers could reactivate 41 and 29% of the aggregated protein, respectively, whereas under nonreducing conditions, these chaperones could reactivate 19 and 25% of the aggregated proteins, respectively. IPMDH was heat-aggregated by incubation at 89 °C for 30 min. Under reducing and nonreducing conditions, wild-type TClpB could reactivate 50 and 48% of the aggregated IPMDH, respectively. Under reducing conditions, the D1 and D2 dimers could reactivate 44 and 38% of the aggregated protein, respectively, whereas under nonreducing conditions, these chaperones could reactivate 32 and 34%, of the aggregated proteins, respectively. In all conditions tested, D1 and D2 dimers showed more than 55% of the disaggregation activity of wild type. In all aspects tested, both D1 and D2 dimers had the essential properties of TClpB and could be used for experiments to evaluate intersubunit cooperativity.
Previously, we demonstrated that the replacement of Lys-Thr with Ala-Ala in the Walker A motifs of AAA-1 (termed 1A) and AAA-2 (termed 2A) inhibited nucleotide binding to the corresponding AAA-/H module (20). To examine the intersubunit cooperativity of TClpB in nucleotide binding, we prepared cross-linked heterodimers containing Walker A-mutated subunits (Table 1). For example, D1 dimers containing subunits having the 1A mutation and subunits having both the 1A and 2A mutations (termed 12A) were prepared by mixing and oxidizing Q184C/A390C and A390C/A390C (termed 1A*12A*D1) or Q184C/A12A*D1 and A390C/1A (termed 12A*1AD1). Similarly, 1A*1AD1 was prepared by oxidizing a mixture of Q184C/1A and A390C/1A. In the AAA-2 rings of the 1A*12A*D1 or 12A*1AD1 hexamers, three intact and three

FIGURE 2. Estimation of the efficiency of intersubunit disulfide bond formation. Cysteine mutants of TClpB were mixed in various combinations as indicated in the figure and oxidized by incubating at 55 °C for 60 min with 50 mM CuCl2 in the absence or presence of 3 mM ATP. Samples were analyzed by SDS-PAGE (6%) in the absence of 2-mercaptoethanol. The arrows indicate the position of the molecular mass marker (MW). A, combinations of cysteine mutants whose mutations were located on the AAA-1 interface are shown. Separately oxidized Q184C and A390C in the absence of ATP are also shown. B–D, combinations of cysteine mutants whose mutations were located on the AAA-2 interface are shown. Oxidation was performed in the absence (B) or presence (C and D) of ATP. Separately oxidized R576C and A821C are also shown (D).

Nucleotide Binding Abilities of the Ordered Heterohexamers—Previously, we demonstrated that the replacement of Lys-Thr with Ala-Ala in the Walker A motifs of AAA-1 (termed 1A) and AAA-2 (termed 2A) inhibited nucleotide binding to the corresponding AAA+ module (20). To examine the intersubunit cooperativity of TClpB in nucleotide binding, we prepared cross-linked heterodimers containing Walker A-mutated subunits (Table 1). For example, D1 dimers containing subunits having the 1A mutation and subunits having both the 1A and 2A mutations (termed 12A) were prepared by mixing and oxidizing Q184C/A390C and A390C/A390C (termed 1A*12A*D1) or Q184C/A12A*D1 and A390C/1A (termed 12A*1AD1). Similarly, 1A*1AD1 was prepared by oxidizing a mixture of Q184C/1A and A390C/1A. In the AAA-2 rings of the 1A*12A*D1 or 12A*1A*D1 hexamers, three intact and three

FIGURE 3. Stabilities of the hexameric structures of D1 and D2 dimers. We analyzed 1.73 μM (as hexamers) wild-type (upper traces), D1 dimer (middle traces), and D2 dimer (bottom traces) TClpB by HPLC size exclusion chromatography at 55 °C in the presence of 2 mM ATP and 150 (dashed line), 500 (thin solid line), or 800 (thick solid line) mM KCl. The arrows indicate the calculated retention time corresponding to 577, 385, and 192 kDa (hexamers, tetramers, and dimers of the 96.2 kDa TClpB subunit, respectively).
Walker A-mutated AAA+ modules were arranged alternately, whereas the AAA-2 ring of the 1A*1AD1 hexamer consisted of six intact AAA+ modules. If the AAA+ modules in AAA-2 ring cooperatively bound nucleotides, the affinities of 1A*12AD1 or 12A*1AD1 for nucleotides would differ from that of 1A*1AD1. When Mant-ADP bound to TClpB, its fluorescence intensity at 440 nm increased. The extent of the increase was plotted against the concentration of Mant-ADP (Fig. 4), and the apparent $K_d$ values were calculated (Table 2). The $K_d$ value for 1A*1AD1 (0.87 μM) was similar to those for 12A*1AD1 (2.7 μM) and 1A*12AD1 (2.3 μM). On the other hand, the $K_d$ value for 2A*2AD1 (12 μM) was similar to those for 12A*2AD1 (16 μM) and 2A*12AD1 (12 μM). We also measured the decreases in Mant-ADP fluorescence by adding Mg-ADP or Mg-ATP, and calculated the apparent $K_d$ values of ADP and ATP for the heterohexamers (Table 2). Although the binding affinities of ADP and ATP were weaker than that of Mant-ADP, a similar tendency was observed. When D2 dimers were used instead of D1 dimers, the results were essentially the same (Table 2).

We tested whether the cross-linked dimers used in the measurement of nucleotide binding could form hexamers using gel filtration analysis (Fig. 5). Although a previous study showed that the hexameric structure of the 1A mutant of TClpB was unstable (20), cross-linked dimers having this mutation could form stable hexamers. This may be related to the stabilization of the hexameric structures of D1 and D2 dimers, as mentioned above. However, some mutants (1A*12AD1, 12A*1AD1, 2A*12AD1, and 2A*12AD2) eluted with a larger molecular mass than the 577-kDa hexamer.

**ATPase Activities of Ordered Heterohexamers**—Next, we prepared cross-linked heterodimers containing subunits having a single Walker A mutation, i.e. 1A*W, W*1A, 1A*1A, 2A*W,
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The ATPase activities of the ordered heterohexamers consist of hydrolyzing ATP at corresponding AAA modules (42). The ordered heterohexamers consisting of wild-type and 1B or 2B subunits were almost equal to those of 1R*1R and 2R*2R, except that the 2R*W D2 showed relatively high ATPase activity (54% of W*W D2).

Like Arg finger mutations, the replacement of Glu with Gln in the Walker B motif of AAA-1 (termed 1B) or AAA-2 (termed 2B) is known to inhibit ATP hydrolysis but not nucleotide binding at the corresponding AAA + module (20). However, unlike Arg finger mutations, the ATPase activities of 1B*1B (45 min⁻¹ for D1 dimer and 30 min⁻¹ for D2 dimer) and 2B*2B (45 min⁻¹ for D1 dimer and 14 min⁻¹ for D2 dimer) were comparable with that of W*W, although the activity of 2B²2B D2 was significantly lower than that of 2B²2B D1 (Fig. 6C). The ordered heterohexamers containing wild-type and 1B or 2B subunits also showed similar ATPase activities (Fig. 6C). Because the Walker B mutation inhibited ATP hydrolysis at the corresponding AAA + module, the ATPase activities of 1B*1B and 2B*2B originate from the intact AAA-2 and AAA-1, respectively. Consistent with this, 12B*12B could not hydrolyze ATP (Fig. 6D).

Moreover, whereas the number of intact AAA + modules was the same, W*12B and 12B*W showed significantly low ATPase activities relative to those of 1B*1B and 2B*2B. This result implied that cooperative ATP hydrolysis occurred in each ring of TClpB. Thus, to evaluate this cooperativity, we prepared cross-linked heterodimers containing subunits having single and double Walker B mutation(s), i.e. 12B*1B, 1B*12B, 12B*2B, and 2B*12B (Table 1), and measured ATPase activities (Fig. 6D). As expected, the ATPase activities of these heterohexamers were significantly lower (less than 14%) than that of W*W.

We tested whether the cross-linked dimers used in the measurement of ATPase activity could form hexamers using gel filtration analysis (Fig. 5). Although W*1A D1 eluted with a larger molecular mass than the 577-kDa hexamer, all other cross-linked dimers eluted at the position of the hexamer, like wild-type protein.

**Disaggregation Activities of Ordered Heterohexamers—** Next, we measured the disaggregation activities of ordered heterohexamers of TClpB using α-glucosidase and IPMDH as substrates (Fig. 7). Similar to 1A*1A and 2A*2A, the ordered heterohexamers consisting of wild-type and Walker A-mutated subunits could not reactivate heat-aggregated substrates (Fig. 7, A and B).

Disaggregation activities of 1R*1R and 2R*2R were very small; however, 26% of aggregated IPMDH was reactivated by 2R²2R D2 (Fig. 7, C and D). When the wild-type subunits were intercalated in the hexamer, the disaggregation activities of Arg finger mutants were restored in part; however, the activities did not reach 50% that of W*W (Fig. 7, C and D).

Next, we found that 1B*1B and 2B*2B reactivated the heat-aggregated α-glucosidase with efficiencies of 20–40% that of W*W (Fig. 7E). Heterohexamers consisting of wild-type and 1B or 2B subunits reactivated the heat-aggregated α-glucosidase, with moderate efficiencies of homohexamers of W*W and 1B*1B or 2B*2B, i.e. 60 – 85% that of W*W. Even when IPMDH was used for a substrate, similar results were observed (Fig. 7F). Homohexamers of double Walker B mutants, i.e. 12B*12B D1 and 12B*12B D2, could not reactivate heat-aggregated substrates, and the intercalation of wild-type subunits to the hexamers had little effect on the restoration of the disaggregation activities (Fig. 7, E and F).

**TABLE 2**

Dissociation constants of nucleotides for TClpB mutants

The values were determined from three to five independent measurements. Standard deviations are shown.

| TClpB | Mant-ADP | ADP | ATP |
|-------|----------|-----|-----|
|        | K_d (μM) |     |     |
| W*W   | 2.8 ± 0.6 | 17 ± 2 | 93 ± 34 |
| 1A*1A | 0.87 ± 0.17 | 7.8 ± 0.8 | 56 ± 1 |
| 12A*1A D1 | 2.7 ± 1.2 | 16 ± 2 | 120 ± 8 |
| 1A*1A D1 | 2.3 ± 1.1 | 19 ± 3 | 180 ± 30 |
| 2A*2A | 12 ± 5 | 9.1 ± 2.0 | 100 ± 38 |
| 1A*1AD2 | 16 ± 3 | 17 ± 7 | 330 ± 86 |
| 2A*2A D1 | 12 ± 5 | 18 ± 12 | 140 ± 66 |
| W*W D2 | 3.0 ± 1.2 | 16 ± 1 | 80 ± 9 |
| 1A*1A D2 | 1.6 ± 1.0 | 12 ± 1 | 110 ± 16 |
| 2A*2A D2 | 3.2 ± 0.7 | 17 ± 3 | 170 ± 23 |
| 1A*1AD2 | 2.2 ± 1.1 | 21 ± 3 | 140 ± 54 |
| 2A*2A D2 | 47 ± 15 | 20 ± 6 | 180 ± 68 |
| 12A*2A D2 | 33 ± 4 | 15 ± 3 | 180 ± 64 |
| 12A*2A D1 | 33 ± 9 | 17 ± 1 | 250 ± 69 |

* The values may only represent the lower limit because of the intrinsic ATPase activity of TClpB.

W*2A, and 2A*2A (Table 1), and measured ATPase activities (Fig. 6A). The ATPase activities of 1A*1A (4.6 min⁻¹ for D1 dimer and 0.0 min⁻¹ for D2 dimer) and 2A*2A (0.6 min⁻¹ for D1 dimer and 3.8 min⁻¹ for D2 dimer) were less than 15% of that of W*W. Such low ATPase activities were barely recovered, even when the wild-type and the Walker A mutant subunits were arranged alternately in the hexamer: at most 12 min⁻¹ (W*1A D1).

Previously, we demonstrated that the Arg finger mutants R322A (termed 1R) and R747A (termed 2R) could bind but not hydrolyze ATP at corresponding AAA + modules (42). The ATPase activities of 1R*1R (6.0 min⁻¹ for D1 dimer and 0.3 min⁻¹ for D2 dimer) and 2R*2R (1.2 min⁻¹ for D1 dimer and 5.9 min⁻¹ for D2 dimer) were less than 22% of W*W (Fig. 6B). The ATPase activities of the ordered heterohexamers consisting of wild-type and 1R or 2R subunits were almost equal to those of 1R*1R and 2R*2R, except that the 2R*W D2 showed relatively high ATPase activity (54% of W*W D2).
In previous studies, researchers showed that the subunits of ClpB functioned cooperatively in the disaggregation process; however, the details of the mechanism were unclear (24, 25, 29). Here, we analyzed the intersubunit cooperativity of the ClpB hexamer using a new approach in which a disulfide bond specifically formed between adjacent subunits. Thirteen TClpB mutants having a cysteine residue on the subunit interface were constructed, and appropriate sets of the mutants were explored to determine their abilities to connect the adjacent subunits by disulfide bonds. Two sets of mutants, Q184C/H11001A390C and R576C/H11001A821C, were found to form disulfide bonds efficiently at the interfaces in the AAA-1 ring and the AAA-2 ring, respectively. Previously, Tsai and co-workers (43) constructed a hexameric ring model of the TClpB AAA-2 module (called D2), based on the newly resolved crystal structure of D2 and the cryo-EM structure of the full-length TClpB. In this model, Arg576 of one subunit faces Ala821 of the adjacent subunit, and the R576C/A821C mutant could form a cross-linked hexamer through a disulfide bond. This finding was consistent with our results. TClpB hexamers consisting of either D1 or D2 dimers were stable and showed significant ATPase and chaperone activities similar to those of the wild type, indicating that the covalent linkage of adjacent subunits through the interfaces on either AAA-1 or AAA-2 did not disrupt the TClpB activity.

Apparent $K_d$ values of various nucleotides for 1A*1A, 1A*12A, 12A*1A, and 1A*12A were approximately the same. Similar results were obtained for 2A*2A, 12A*2A, and 2A*12A. These results indicated that the nucleotide binding-defective domain did not affect nucleotide binding to the surrounding domains in the same ring. Previously, Muga and co-workers (27) tested the nucleotide binding properties of the AAA-1 and AAA-2 rings of E. coli ClpB (EClpB) using indirect (limited proteolysis) and direct (isothermal titration calorimetry) methods, respectively, and suggested that there was no intraring cooperativity for nucleotide binding to these rings. Our results were consistent

![FIGURE 5. Stabilities of the hexameric structures of TClpB mutants.](image_url)
with this observation. On the other hand, the substantial differences in the affinities of nucleotides between AAA-1 and AAA-2 found in E. Coli were not observed in T. Coli (27). More-
This was also supported by the observation that the ATPase activities of the ordered heterohexamers consisting of wild-type and 12B subunits were significantly lower than those of 1B*1B and 2B*2B, despite the fact that the numbers of intact AAA\textsubscript{H11001} modules were the same. This result was consistent with a previous observation that the simple doping of double Walker B-mutated subunits decreased the ATPase activity of wild-type T\textsubscript{ClpB} (24).

In summary, our results indicated that the ATPase cycle of T\textsubscript{ClpB} proceeded as follows: (i) the 12 AAA\textsubscript{H11001} modules ran-

FIGURE 7. Chaperone activities of the T\textsubscript{ClpB} mutants. α-Glucosidase (A, C, and E) or IPMDH (B, D, and F) (final concentration, 0.2 µM monomers) was incubated at 73 °C for 10 min or 89 °C for 30 min, respectively, in the presence of 3 mM ATP. The temperature was shifted down to 55 °C, and T\textsubscript{DnaK} (0.6 µM), T\textsubscript{DnaJ} (0.2 µM), T\textsubscript{GrpE} (0.1 µM dimer), and T\textsubscript{ClpB} mutants (0.05 µM hexamer) were added immediately to the reaction mixture. After incubation at 55 °C for 90 min, the recovered enzyme activity was measured. The enzyme activities recovered by the T\textsubscript{ClpB} variants of homohexamers and heterohexamers consisting of wild-type and single Walker A-mutated subunits (A and B), wild-type and single Arg finger-mutated subunits (C and D), and wild-type and single and double Walker B-mutated subunits (E and F) are shown. The enzyme activities recovered by the W*W were set as 100%. The error bars represent standard deviations of three independent measurements.
domly bound ATP, (ii) the binding of four or more ATP to one AAA + ring was sensed by the Arg finger and converted another AAA + ring into the ATPase-active form, and (iii) ATP hydrolysis occurred cooperatively in each ring (Fig. 8). Recently, Saibil and co-workers (45) showed that each AAA + ring of the ClpB hexamer simultaneously binds four ADP molecules. From this observation, the binding of four ATP molecules to one ring would be expected to activate the ATPase of another ring.

In contrast to TClpB, ATPase activities of ClpB and Hsp104 were increased by doping of double Walker B-mutated subunits, suggesting that step (iii) above did not fit with these proteins (25, 26, 28, 29). Conversely, Walter and co-workers (28) performed more precise doping experiments using single and double Walker B-mutated Hsp104 and suggested that cooperative ATP hydrolysis occurred in the AAA-1 ring of Hsp104. Further studies are required to clarify whether step (iii) is a common feature of ClpB and Hsp104. For example, if our method applied to ClpB and Hsp104, the conclusions might change because the same subunits would not adjoin in the heterohexamers prepared using our method, different from the simple doping method.

Although most of the homohexamers of Walker A mutants or Arg finger mutants could not reactivitate aggregated substrates, homohexamers of single Walker B mutants showed low but significant disaggregation activities. These results implied that the disaggregation process required at least one AAA + ring preceding cooperative ATP hydrolysis. Consistent with this assumption, the heterohexamers consisting of wild-type and single Walker A-mutated subunits or wild-type and double Walker B-mutated subunits could barely reactivitate the aggregated substrates. In contrast, some of the heterohexamers containing wild-type and Arg finger-mutated subunits showed significant disaggregation activity. Because some of these heterohexamers have residual ATPase activity, intersubunit communications through amino acid residues other than the Arg finger residues may compensate for the lack of an Arg finger to some extent.

Through intercalation of three wild-type subunits, the disaggregation activities of single Walker B mutants increased and reached to about midpoint between the activities of the mutant and wild-type proteins, regardless of the increase or decrease in ATPase activity. These results suggested that the disaggregation activity correlated with the number of intact AAA + modules if cooperative ATP hydrolysis occurred in more than one ring.

In size exclusion chromatography, five cross-linked dimers (W*1AD1, 1A*12AD1, 12A*1AD1, 2A*12AD1, and 2A*12AD2) eluted faster than the position of the TClpB hexamer. These dimers may form some nonspherical structure or higher order oligomer, although the exact reason was not known. In any case, in all combinations of mutant or wild-type subunits, there were at least two synonymous cross-linked dimers that could form stable hexamers, like wild-type protein. Thus, the conclusions of this manuscript were not influenced.

In some cases, hexamers consisting of the same set of mutant or wild-type subunits showed different activities depending on the conjugation method: cross-linking at D1 or D2 and the arrangement of the subunits in cross-linked dimers. For example, the ATPase activity of W*1AD1 was higher than those of other heterohexamers consisting of 1A and wild-type subunits. Similarly, the ATPase activity of 2R*W D2 was higher than those of the other heterohexamers having the same set of subunits. In these heterohexamers, disulfide bonds were formed near the nucleotide binding pockets, which were influenced by the introduced mutations. Although we cannot exclude the possibility that the disulfide cross-linking partly absorbed the effects of the mutation, the correctness of the interpretations pro-

**FIGURE 8. Model for the ATPase cycle of TClpB.** The AAA + modules that bound ATP are shown in gray. The AAA + rings proceeding cooperative ATP hydrolysis are shown as a striped pattern.
posed in this manuscript was guaranteed by the other samples whose disulfide bonds were formed in other positions. Beyond that, the ATPase activity of 2B\textsuperscript{2BD12} was twice as high as that of 2B\textsuperscript{2BD2}. Based on the ATPase activity of 2B, which had no disulfide bond (14 min\textsuperscript{-1}), the activity of 2B\textsuperscript{2BD12} would be sufficient. The exact reason for this discrepancy is not known; however, both 2B\textsuperscript{2BD12} and 2B\textsuperscript{2BD2} had significant ATPase activities, and there was little effect on our interpretations.

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