

Coupling ATP utilization to protein remodeling by ClpB, a hexameric AAA+ protein

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ClpB and Hsp104 are members of the AAA+ (ATPases associated with various cellular activities) family of proteins and are molecular machines involved in thermotolerance. They are hexameric proteins containing 12 ATP binding sites with two sites per protomer. ClpB and Hsp104 possess some innate protein remodeling activities; however, they require the collaboration of the DnaK/Hsp70 chaperone system to disaggregate and reactivate insoluble aggregated proteins. We investigated the mechanism by which ClpB couples ATP utilization to protein remodeling with and without the DnaK system. When wild-type ClpB, which is unable to remodel proteins alone in the presence of ATP, was mixed with a ClpB mutant that is unable to hydrolyze ATP, the heterohexamers surprisingly gained protein remodeling activity. Optimal protein remodeling by the heterohexamers in the absence of the DnaK system required approximately three active and three inactive protomers. In addition, the location of the active and inactive ATP binding sites in the hexamer was not important. The results suggest that in the absence of the DnaK system, ClpB acts by a probabilistic mechanism. However, when we measured protein disaggregation by ClpB heterohexamers in conjunction with the DnaK system, incorporation of a single inactive ClpB subunit blocked activity, supporting a sequential mechanism of ATP utilization. Taken together, the results suggest that the mechanism of ATP utilization by ClpB is adaptable and can vary depending on the specific substrate and the presence of the DnaK system.

DnaJ | DnaK | GrpE | protein disaggregation

Bacterial ClpB and yeast Hsp104 are ATP-dependent protein remodeling machines that function to disaggregate protein aggregates and reactivate proteins after extreme stress conditions (1–3). In the cell, ClpB acts in conjunction with the DnaK chaperone system and Hsp104 acts with the Hsp70 chaperone system (4, 5). DnaK and Hsp70 are members of another large, ubiquitous family of ATP-dependent molecular chaperones that mediate protein reactivation and remodeling in concert with two cochaperones, DnaJ and GrpE in prokaryotes and Hsp40 and NEF in eukaryotes (6). Alone, neither the DnaK/Hsp70 chaperone system nor ClpB/Hsp104 has the ability to reactivate large insoluble aggregates.

ClpB/Hsp104 exists as a hexameric ring with an axial channel (7–10). Each protomer contains two AAA+ (ATPases associated with various cellular activities) nucleotide-binding domains separated by a hinge region and preceded by an N-terminal domain (1, 7). The two AAA+ domains contain characteristic motifs, including Walker A and B and sensor-1 and -2 motifs, as well as an arginine finger (11, 12). Situated in the first AAA+ domain is a long coiled-coil region, referred to as the middle domain, which is unique to ClpB, Hsp104, and their homologs.

In vitro ClpB/Hsp104 solubilizes and reactivates protein aggregates in ATP-dependent reactions in collaboration with the DnaK/Hsp70 chaperone system (1–3). Although the roles of the two chaperone systems in disaggregation are not fully understood, it is likely that ClpB/Hsp104 is the primary protein disaggregating machine, and DnaK/Hsp70 facilitates the interaction of ClpB/Hsp104 with aggregates (1–3). However, DnaK/Hsp70 may have additional functions.

Evidence suggests that ClpB/Hsp104 acts by forcibly extracting polypeptides from aggregates and translocating the unfolded regions of polypeptides through its axial channel (1–3). This substrate translocation mechanism has been established for ClpA and ClpX, two Clp ATPases that interact with a proteolytic component, ClpP (13). Substrates are unfolded and threaded through the central channels of ClpA and ClpX directly into the chamber of ClpP where degradation occurs. Work from Bukau and colleagues, in which ClpB and Hsp104 were engineered to contain the ClpP interaction loop from ClpA, supports a similar mechanism for ClpB and Hsp104 (14, 15).

The demonstration that ClpB and Hsp104 have an innate ability to unfold and activate proteins in the absence of the DnaK/Hsp70 chaperone system provides additional support for an unfolding and translocation mechanism (16). However, ATP alone is ineffective in promoting these protein remodeling activities. Instead, mixtures of ATP and ATP γ S, a slowly hydrolyzed ATP analog, are required for remodeling, suggesting that with the mixture of nucleotide substrates can be held (a function requiring ATP binding and supported by ATP γ S) and unfolded and translocated (functions requiring ATP hydrolysis).

To understand how ClpB couples ATP binding and hydrolysis to protein remodeling, we investigated the contribution of the 12 ATP binding sites to the overall chaperone activity of ClpB both alone and in the presence of the DnaK chaperone system.

Results

ClpB Hexamers with a Balance of Active and Inactive Nucleotide Binding Sites Are Required for Optimal Protein Remodeling in the Absence of the DnaK System. The 12 ATP binding sites of hexameric ClpB are arranged in two rings of six, with each protomer providing one nucleotide-binding site to each ring, referred to here as Ring-1 and Ring-2 (Fig. 1A). Ring-1 of the hexamer is comprised of the N-terminal ATP binding sites, and Ring-2 is comprised of the C-terminal ATP-binding sites.

Previous observations demonstrated that ATP hydrolysis at <12 ATP binding sites of the ClpB hexamer is required to elicit the innate protein remodeling activity of ClpB, and ATP hydrolysis at all 12 sites prohibits activity (16). In the work presented here, we used a green fluorescent protein (GFP) fusion protein containing a C-terminal 15 aa peptide, GFP-15, as a substrate for remodeling. We measured protein unfolding by monitoring the decrease in fluorescence with time in the presence of a mutant GroEL (17) that binds unfolded proteins but does not release them. ClpB wild-type, ClpB_(wt), was unable to unfold GFP-15 in the presence of ATP (Fig. 1B). However, when mixtures of ATP and ATP γ S were used in a 1:1 ratio, there was a large decrease in GFP fluorescence (Fig. 1C). Thus, if some

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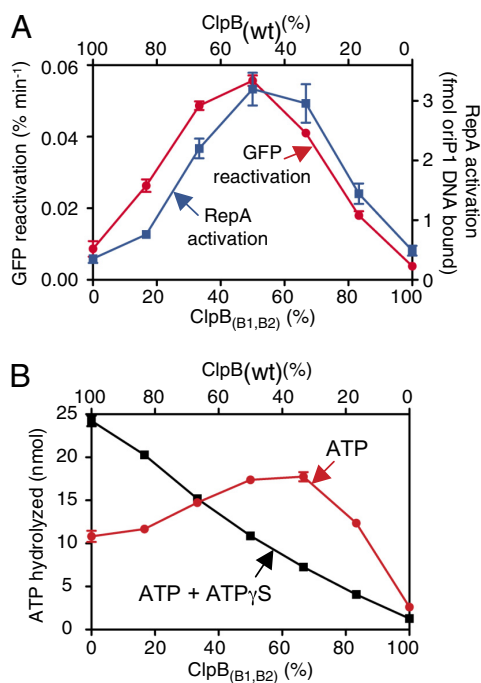


Fig. 2. ClpB hexamers containing approximately six active ATP hydrolytic sites are required for optimal chaperone activity. (A) Reactivation of heat-aggregated GFP and activation of RepA was measured as described in the *Methods* using various ratios of ClpB_(wt) to ClpB_(B1,B2). GFP reactivation rates (left axis) and DNA binding by RepA (right axis) were plotted as a function of percent ClpB_(B1,B2). (B) ATPase activity by mixtures of ClpB_(wt) and ClpB_(B1,B2) in the presence of ATP or ATP and ATP γ S in a 1:1 ratio was measured as described in the *Methods*. ATPase activity is plotted as a function of percent ClpB_(B1,B2). Data are means \pm SEM ($n = 3$). Some error bars are covered by plot symbols.

observed with a 1:1 ratio of the two ClpB proteins. Mixtures of ClpB_(B1,B2) and ClpB_(wt) were also tested in the RepA activation assay in which inactive RepA dimers are converted to monomers that bind DNA with high affinity (22). In this assay as well, maximal activation was seen with a 1:1 mixture of the two proteins (Fig. 2A). Thus, in additional protein remodeling reactions, heterohexamers of ClpB_(B1,B2) and ClpB_(wt) are functional, while neither protein separately exhibits significant activity.

When ATP hydrolysis was measured, approximately 2-fold higher steady-state hydrolysis was observed with a 1:1 mixture of ClpB_(wt) and ClpB_(B1,B2) compared to ClpB_(wt) alone (Fig. 2B) (19). This suggests that the activity of wild-type subunits within the heterohexamer is stimulated. For example, at a 1:1 ratio of ClpB_(wt) to ClpB_(B1,B2), the ATPase activity of the wild-type subunits must increase approximately 4-fold to account for the observed increase in ATP hydrolysis. For comparison, when we measured ATP hydrolysis by mixtures of ClpB_(wt) and ClpB_(B1,B2) in the presence of ATP and ATP γ S, hydrolysis decreased linearly as the percent of ClpB_(B1,B2) in the mixture was increased (Fig. 2B). ATP hydrolysis by ClpB_(wt) alone was approximately 2-fold higher with a 1:1 mixture of ATP and ATP γ S than with ATP alone (Fig. 2B) (16). Thus, conditions optimal for protein remodeling are also optimal for ATP hydrolysis.

Our results with the single Walker B mutants and heterohexamers of ClpB_(wt) and ClpB_(B1,B2) suggest that for remodeling activity by ClpB alone: (i) protomers need not contain a pair of active sites as long as there are a total of six active sites in either Ring-1 or Ring-2 of the hexamer, and (ii) there need not be six functional sites in a single ring as long as there are approximately six active sites in the hexamer. Together these results indicate

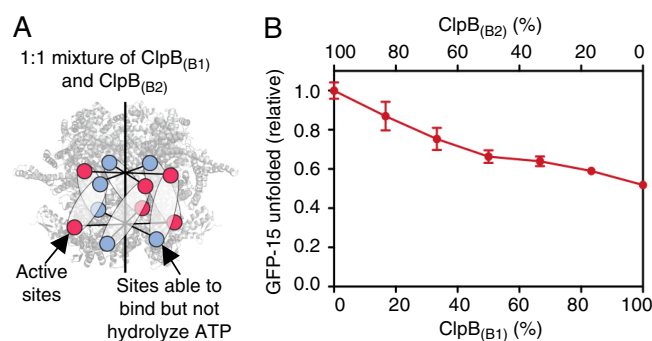


Fig. 3. Protein unfolding by mixtures of ClpB_(B1) and ClpB_(B2). (A) Diagram showing a heterohexamer containing ClpB_(B1) and ClpB_(B2) in a 1:1 ratio. (B) GFP-15 unfolding by mixtures of ClpB_(B1) and ClpB_(B2) was measured as described in the *Methods*. Rates are expressed as a fraction relative to ClpB_(B2) alone ($0.060 \pm 0.002 \text{ min}^{-1}$) and plotted as a function of percent ClpB_(B1) in the mixture. Data are means \pm SEM ($n \geq 3$). Some error bars are covered by plot symbols.

that the innate remodeling activity of ClpB might simply require a total of approximately six active ATP hydrolytic sites, independent of the location of the sites within the subunit or within the hexamer.

To test this possibility, we measured protein unfolding by heterohexamers of the two single Walker B mutants, ClpB_(B1) and ClpB_(B2) (Fig. 3A). When ClpB_(B1) and ClpB_(B2) were mixed in various ratios while keeping the total concentration of ClpB constant, there was no significant gain or loss of function (Fig. 3B). Interpretation of these results depends upon ClpB_(B1) and ClpB_(B2) forming heterohexamers, which is likely, since each single Walker B mutant is capable of forming heterohexamers with both ClpB_(wt) and ClpB_(B1,B2) (shown in Fig. 4B and D and Fig. 5B). The results suggest that ClpB is active in protein remodeling when the hexamer contains six active ATP hydrolytic sites irrespective of the position of the active sites within the hexamer.

Mechanism of ATP Utilization by ClpB in the Absence of the DnaK System. Our observations that heterohexamers of ClpB_(wt) and ClpB_(B1,B2) perform remodeling activities demonstrate that protein unfolding and remodeling can be carried out with approximately three active protomers per hexamer (Figs. 1 and 2). These results rule out a concerted mechanism of ATP utilization within a ring, where all six active sites of a ring simultaneously bind ATP, hydrolyze ATP, and release ADP. They also rule out a strictly sequential mechanism, where activity depends upon an endless cycle of ATP utilization around the ring. Instead, the observations point to either a probabilistic mechanism, involving a random order of mutually independent hydrolysis events or a semisequential mechanism, in which the sequence and timing of ATP hydrolysis operates in a sequential manner that proceeds around the ring with hydrolysis pausing occasionally and then resuming at another site in the ring.

To further explore the mechanism of ATP utilization by ClpB, we varied the number of active sites in one ring while maintaining six inactive sites in the other ring. We first decreased the number of active sites in Ring-2 by increasing the percentage of ClpB_(B1,B2) in mixtures with ClpB_(B1) (Fig. 4A). We observed that incorporation of one or two inactive sites in Ring-2 had little effect on hexamer activity, while incorporation of four or five inactive sites significantly inhibited activity (Fig. 4B). We next changed the number of active sites in Ring-1 by varying the ratio of ClpB_(B1,B2) in mixtures with ClpB_(B2) (Fig. 4C) and observed similar results (Fig. 4D). Taken together, these experiments show that ClpB is able to function in protein remodeling with one

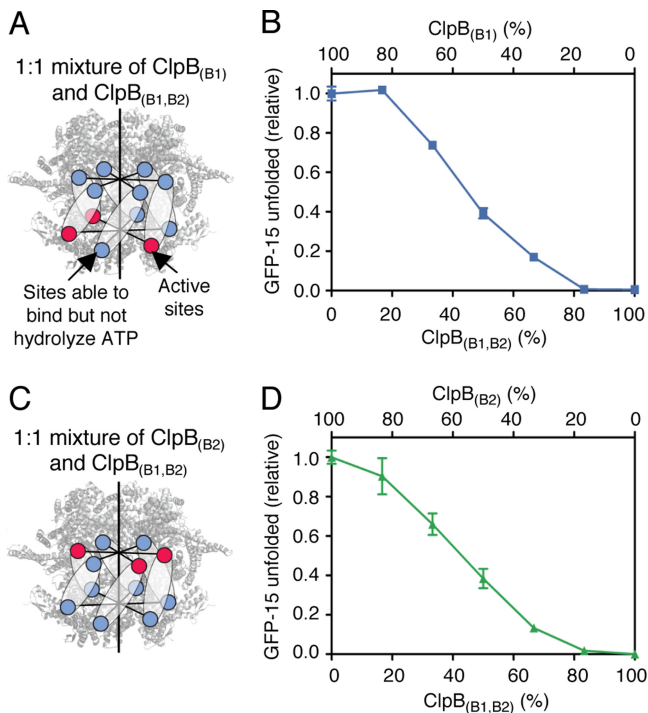


Fig. 4. Protein unfolding by mixtures of ClpB_(B1,B2) and either ClpB_(B1) or ClpB_(B2). (A and C) Diagrams showing heterohexamers of ClpB_(B1,B2) with ClpB_(B1) (A) or ClpB_(B2) (C) in a 1:1 ratio. (B and D) GFP-15 unfolding by mixtures of ClpB_(B1,B2) and ClpB_(B1) (B) or ClpB_(B2) (D) as described in the *Methods*. Unfolding rates are expressed as a fraction relative to ClpB_(B1) ($0.035 \pm 0.001 \text{ min}^{-1}$) (B) or ClpB_(B2) ($0.060 \pm 0.002 \text{ min}^{-1}$) (D) alone and plotted as a function of percent ClpB_(B1,B2) in the mixture. Data are means \pm SEM ($n \geq 3$). Some error bars are hidden by plot symbols.

inactive ring and approximately three or more active sites in the other ring of the hexamer (Fig. 4 B and D). Thus, the results best support a probabilistic or a semisequential mechanism of ATP utilization by the ClpB rings. However, six active sites per hexamer are optimal for protein remodeling in the absence of the DnaK system (Figs. 1 F and G and 2–4).

Mechanism of ATP Utilization by ClpB When Disaggregating Substrates That Require the DnaK System. To test whether the mechanism of ATP utilization by ClpB is the same when ClpB works on aggregates with the DnaK system as when it works alone, we monitored disaggregation by mixtures of ClpB_(wt) and ClpB_(B1,B2) in conjunction with DnaK, DnaJ, and GrpE. We used aggregated malate dehydrogenase (MDH) as a substrate, since previous studies showed that MDH disaggregation by ClpB in combination with the DnaK system was approximately 20-fold greater than disaggregation by either chaperone alone (23, 24). As the percent of ClpB_(B1,B2) in mixtures with ClpB_(wt) was increased, we observed a rapid exponential decrease in MDH disaggregation, compared to the linear decrease expected if a probabilistic mechanism was used (Fig. 5A). The loss of activity when the percentage of ClpB_(B1,B2) is low indicates that incorporation of approximately one protomer with two hydrolytically defective ATP binding sites blocks disaggregation of MDH. Our results indicate a striking departure from the protein remodeling and ATP-ase activities seen in the absence of the DnaK system (Figs. 1 F and G and 2 A and B) and from the ATPase data (Fig. 2B). They suggest that for disaggregation of protein aggregates in collaboration with the DnaK system, cooperative interactions between all six ClpB subunits are required. Similar inter-subunit coupling during remodeling has been observed for *Thermus*

thermophilus ClpB using another substrate, aggregated α -glucosidase (20).

We next measured the effects of single nucleotide binding site mutants in mixtures with ClpB_(wt). We used the two single Walker B mutants discussed above, ClpB_(B1) and ClpB_(B2). In addition, we tested a ClpB Walker A mutant, ClpB_(A2), with a K611T substitution in Ring-2 that renders it unable to bind ATP in Ring-2.* All three mutants are defective in disaggregation of MDH in combination with the DnaK system (23, 25) (Fig. 5 B and C). As the percentage of ClpB_(B1), ClpB_(B2), or ClpB_(A2) in mixtures with ClpB_(wt) was increased, disaggregation of MDH decreased more rapidly than the linear decrease expected for a probabilistic mechanism of action (Fig. 5 B and C). However, incorporating approximately two inactive sites in the same ring inhibited disaggregation less than incorporating approximately one protomer with two inactive sites (Fig. 5 A and B). These observations suggest that for ClpB to work on substrates that require collaboration with the DnaK system, both inter- and intra-ring communication between ClpB subunits is important. Together the data support a sequential or semisequential mechanism of ATP utilization within each ring and within the hexamer. Moreover, they imply that the mechanism of ATP utilization by ClpB can vary depending on the substrate and the requirement for the DnaK system.

To address the question of whether the specific aggregate influences ATP utilization requirements, we compared disaggregation of aggregated MDH to that of heat-aggregated GFP-38, a GFP fusion protein containing a C-terminal 38 aa peptide. Aggregated GFP-38, like MDH, required the combination of ClpB_(wt) and the DnaK system for reactivation, as measured by the regain of GFP fluorescence with time (Fig. 6A). ClpB_(B1) and ClpB_(B2) were each able to reactivate GFP-38 at a low rate in the presence of the DnaK system, although neither mutant was able to detectably disaggregate MDH (Figs. 5B and 6A). These results suggest that with some aggregates, six active ATP binding sites are sufficient to carry out limited disaggregation in conjunction with the DnaK system.

To extend the comparison of the two aggregates, we measured disaggregation of GFP-38 by mixtures of ClpB_(B1,B2) and ClpB_(wt). Disaggregation activity decreased exponentially as the ratio of ClpB_(B1,B2) to ClpB_(wt) in the mixture increased (Fig. 6B). The data provide further support that cooperative interactions between subunits are important and that ATP utilization by ClpB is likely through a sequential or semisequential mechanism when ClpB acts in protein disaggregation in conjunction with the DnaK system.

In summary the results presented here suggest that the mechanism of ATP utilization by the two rings of ClpB can change dependent upon the specific substrate and the requirement for the DnaK chaperone system.

Discussion

The surprising result that the heterohexamer of ClpB_(wt) and ClpB_(B1,B2) is active under conditions where neither homohexamer is active shows that modulation of the hydrolytic cycle of ClpB elicits protein remodeling. Moreover, approximately six active nucleotide-binding sites are required for maximal protein remodeling activity by ClpB alone (Figs. 1 and 2). These observations are consistent with our previous findings that ClpB and Hsp104 perform protein remodeling alone in the presence of mixtures of ATP and ATP γ S (16). Together the data suggest that remodeling activity is elicited when some sites hydrolyze ATP and other sites bind ATP, but hydrolyze

*Unlike a Walker A mutant with a K611T substitution in Ring-2, a Walker A mutant with a substitution in Ring-1, K212T, is unable to form hexamers (22, 27). For this reason, ClpB_(K212T) and a double Walker A mutant were not used.

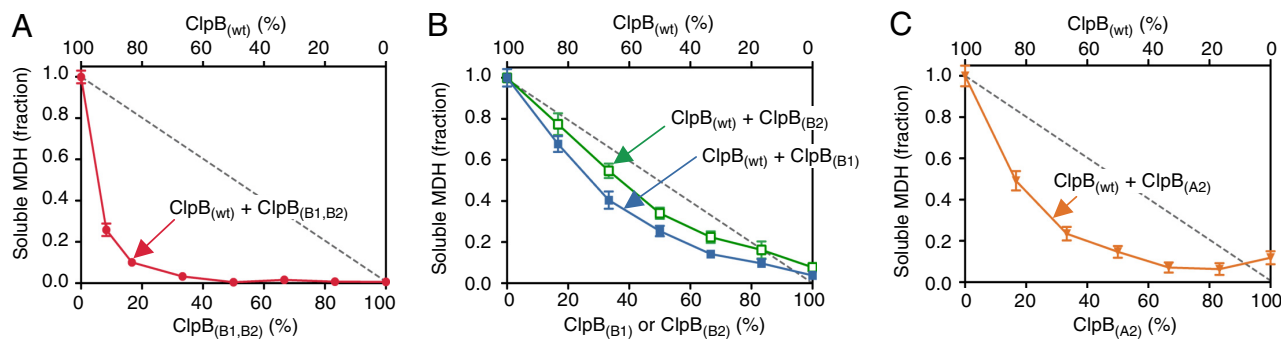


Fig. 5. Disaggregation of MDH by mixtures of ClpB_(wt) and ClpB mutants in conjunction with DnaK, DnaJ, and GrpE. (A–C) Recovery of soluble MDH from heat-induced aggregates was measured as described in the *Methods* for mixtures of ClpB_(wt) with ClpB_(B1,B2) (A), ClpB_(B1) (B), ClpB_(B2) (B), or ClpB_(A2) (C) in the presence of DnaK/DnaJ/GrpE. In A–C, the dashed gray line represents the linear decrease expected for a probabilistic mechanism where the activity of the hexamer is proportional to the number of wild-type subunits. The fraction of soluble MDH recovered in reactions containing 100% ClpB_(wt) was set equal to one. With conditions used, ClpB_(wt) solubilized 53% ± 1% of the MDH. Data are means ± SEM ($n \geq 3$). Some error bars are covered by plot symbols.

it slowly or not at all. One interpretation is that the sites that bind but do not hydrolyze ATP help stabilize interactions between ClpB and the substrate as well as interactions among ClpB protomers in the hexamer, while the sites that hydrolyze ATP are necessary for substrate unfolding and translocation. The balance between the two types of sites influences the efficiency of protein remodeling. However, modulating the ATP hydrolytic cycle is not sufficient to elicit the full activity of ClpB, since *in vivo*, mutants defective in ATP hydrolysis in either ring are also defective for thermotolerance (18). One possibility is that the substrate and the DnaK system regulate

ATP utilization, consistent with our previous results showing that ATP hydrolysis is stimulated by the combined presence of ClpB, the DnaK system, and aggregated substrate (23).

Interestingly, our results suggest that the number of hydrolytically active sites sufficient to perform remodeling activity and the mechanism of ATP utilization can vary depending on the substrate and the involvement of the DnaK system. When ClpB acts in the absence of the DnaK system, the mechanism of ATP utilization by the two rings is likely probabilistic, although a semisequential mechanism is also possible. This is based on experiments showing that approximately three active protomers per hexamer are optimal for remodeling activity. In addition, approximately three active ATP hydrolytic sites in a ring are sufficient for protein remodeling when the other ring contains six sites defective in ATP hydrolysis. In contrast, when ClpB acts on aggregated substrates that require the DnaK system, ClpB protomers must work together in a cooperative fashion, supporting a sequential mechanism of ATP utilization. However, the observations that heterohexamers can tolerate protomers with one active and one inactive ATP binding site provide evidence for a semisequential mechanism of ATP utilization within the separate rings.

Probabilistic, semisequential, and sequential modes of action have been proposed for other AAA+ proteins (10, 26, 27). For example, another hexameric Clp protein, ClpX, has been shown to function in a probabilistic manner by Martin, Baker, and Sauer (26). In contrast, Saibil, Lindquist, and colleagues recently proposed that Hsp104, the yeast homolog of ClpB, uses a sequential mechanism, based on electron microscopic data showing asymmetry in the hexameric model (10). Crystal structures showing asymmetric conformations have been observed for several AAA+ helicases, suggesting that those proteins act by a sequential mechanism (27). Additionally, subunit mixing studies provided evidence for a semisequential mechanism of action by MCM and RuvB and a sequential mechanism for T7gp4 (28–32).

While further biochemical and structural analyses of ClpB and its interaction with the DnaK system are needed, the mechanistic insights revealed by this study of ClpB may extend to Hsp104 and other ClpB homologs.

Methods

Proteins and DNA. P1 RepA (22), GFP (33), GFP-15 (34), ClpB and ClpB mutants (35), GroEL_{trap} (17), DnaK (36), DnaJ (36), GrpE (36), and [³H]JoriP1 DNA (22) (4,475 cpm/fmol) were prepared as described. pET-GFP-38 was created by inserting GAT and GAC codons before the multicloning site stop codon of pET-GFP-X₃₀-H₆ (37) by QuikChange (Stratagene) mutagenesis. GFP-38 was purified as described for GFP-X₃₀-H₆ (37). MDH (Roche) was labeled with ³H as

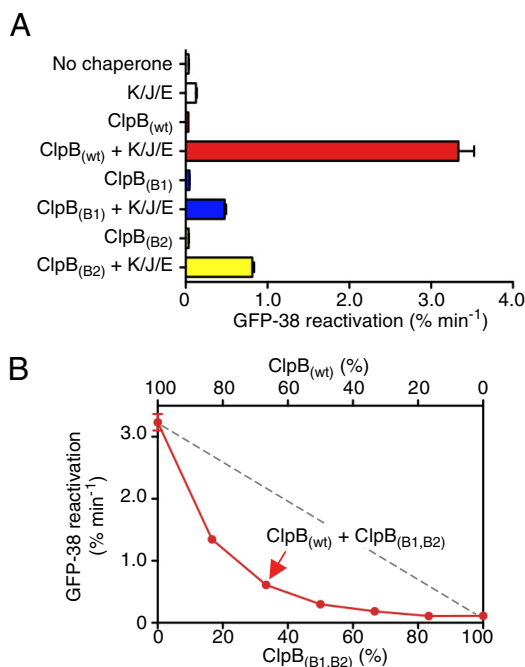


Fig. 6. Disaggregation of GFP-38 by mixtures of ClpB_(wt) and ClpB mutants in conjunction with DnaK, DnaJ, and GrpE. (A) The rate of reactivation of heat-inactivated GFP-38 by ClpB_(wt) or ClpB Walker B mutants was measured with and without DnaK/DnaJ/GrpE as described in the *Methods*. (B) The rate of GFP-38 reactivation by mixtures of ClpB_(wt) and ClpB_(B1,B2) with DnaK/DnaJ/GrpE was measured as described in the *Methods* and plotted as a function of percent ClpB_(B1,B2). The dashed gray line represents the linear decrease expected for a probabilistic mechanism where the activity of the hexamer is proportional to the number of wild-type subunits. Data are means ± SEM ($n = 3$). Some error bars are hidden by plot symbols.

described in ref. 38. Protein concentrations given are for monomeric GFP, GFP-15, GFP-38, MDH, and DnaK; dimeric RepA, DnaJ, and GrpE; hexameric ClpB; and tetradecameric GroEL_{trap}.

Assays. GFP-fusion protein unfolding (16), GFP reactivation (16), MDH disaggregation (23), RepA activation (16), and ATPase (16) assays were performed as described with slight modifications described in the *SI Methods*. GFP-38 reactivation was performed as described in the *SI Methods*. For subunit mixing experiments, control experiments were conducted to demonstrate that the activity of

ClpB_(wt), ClpB_(B1), and ClpB_(B2) decreased linearly upon dilution over the range of concentrations used.

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