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Interplay between *E. coli* DnaK, CIpB and GrpE during protein disaggregation

Shannon M. Doyle^a, Shankar Shastry^a, Andrea N. Kravats^a, Yu-Hsuan Shih^b, Marika Miot^{a,c}, Joel R. Hoskins^a, George Stan^b, and Sue Wickner^{a,§}

^aLaboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

^bDepartment of Chemistry, University of Cincinnati, Cincinnati, OH 45221, USA

Abstract

The DnaK/Hsp70 chaperone system and ClpB/Hsp104 collaboratively disaggregate protein aggregates and reactivate inactive proteins. The teamwork is specific: E. coli DnaK interacts with E. coli ClpB and yeast Hsp70, Ssa1, interacts with yeast Hsp104. This interaction is between the M-domains of hexameric ClpB/Hsp104 and the DnaK/Hsp70 nucleotide-binding domain (NBD). To identify the site on E. coli DnaK that interacts with ClpB, we substituted amino acid residues throughout the DnaK NBD. We found that several variants with substitutions in subdomain IB and IIB of the DnaK NBD were defective in ClpB interaction in vivo in a bacterial two-hybrid assay and in vitro in a fluorescence anisotropy assay. The DnaK subdomain IIB mutants were also defective in the ability to disaggregate protein aggregates with ClpB, DnaJ and GrpE, although they retained some ability to reactivate proteins with DnaJ and GrpE in the absence of ClpB. We observed that GrpE, which also interacts with subdomains IB and IIB, inhibited the interaction between ClpB and DnaK in vitro, suggesting competition between ClpB and GrpE for binding DnaK. Computational modeling of the DnaK-ClpB hexamer complex indicated that one DnaK monomer contacts two adjacent ClpB protomers simultaneously. The model and the experiments support a common and mutually exclusive GrpE and ClpB interaction region on DnaK. Additionally, homologous substitutions in subdomains IB and IIB of Ssa1 caused defects in collaboration between Ssa1 and Hsp104. Altogether, these results provide insight into the molecular mechanism of collaboration between the DnaK/Hsp70 system and ClpB/Hsp104 for protein disaggregation.

Keywords

GrpE; nucleotide exchange factor; Hsp40; protein disaggregation; molecular chaperone

[§]To whom correspondence should be addressed: Sue Wickner, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, 37 Convent Drive, Bldg. 37, Room 5144, Bethesda, MD, 20852, USA Tel.: (301) 496-2629; Fax: (301) 402-1344; wickners@mail.nih.gov.

^CCurrent Address: Telomere and Genome Stability Laboratory, Instituto Gulbenkian de Ciência, Oeiras 2781-901, Portugal.

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Introduction

The Hsp70 family of ATP-dependent molecular chaperones are involved in a multitude of protein activation and remodeling reactions including protein folding, protein transport and protein disaggregation ^{1–3}. Hsp70 proteins are able to perform this variety of activities because they can collaborate with many additional chaperones and co-chaperones. *Escherichia coli* Hsp70, DnaK, collaborates with an Hsp40 protein, DnaJ, and a nucleotide exchange factor (NEF), GrpE ^{1–3}. DnaJ recruits DnaK to the substrate and stimulates ATP hydrolysis by DnaK, which promotes stable substrate binding ⁴. GrpE stimulates ADP and substrate release, priming DnaK for another round of substrate binding ⁵. DnaK has also been shown to collaborate with additional ATP dependent chaperones, including *E. coli* Hsp90 and ClpB ^{6–8}.

DnaK is comprised of two domains, the N-terminal nucleotide-binding domain (NBD) and the C-terminal substrate-binding domain (SBD) (Fig. 1A) ^{9, 10}. The NBD is defined by four subdomains: IA, IIA, IB and IIB (Fig. 1A and B). Co-chaperones DnaJ and GrpE have been shown to directly interact with the DnaK NBD (Fig. 1B) ^{2, 3, 11–13} to regulate nucleotide binding, hydrolysis and exchange, processes which in turn are coupled to substrate binding and release ^{2, 3}. Large conformational changes in DnaK accompany the binding and hydrolysis of ATP by DnaK ^{2, 3, 14–20}. The ADP-bound closed conformation promotes stable interactions with substrate, while the ATP-bound open conformation binds weakly to substrates ^{14–20}.

The DnaK system has been shown to collaborate both in vivo and in vitro with ClpB for disaggregation and reactivation of insoluble protein aggregates ^{7, 8, 21}. This collaboration is specific: E. coli DnaK (DnaK_{Ec}) collaborates with E. coli ClpB (ClpB_{Ec}), yeast Hsp70 collaborates with yeast Hsp104 and Thermus thermophilus DnaK (DnaK_{Tth}) functions with T. thermophilus ClpB (ClpB_{Tth}) $^{21-23}$. A recent in vitro study using multiple nuclear magnetic resonance (NMR) spectroscopy techniques, reported an atomic-resolution model for the ClpB-DnaK complex from T. thermophilus ²⁴. To identify residues involved in the intermolecular interaction, monomers and monomeric fragments of both ClpB_{Tth} and DnaK_{Tth} were utilized ²⁴. Results were consistent with previously described experiments using ClpB_{Ec} and Hsp104, and localized the DnaK_{Tth} interaction site on ClpB_{Tth} to residues in helices 2 and 3 of the M-domain (Fig. 1C) ^{24–27}. The ClpB_{Tth} interaction site on DnaK_{Tth} was mapped to subdomains IB and IIB of the DnaK_{Tth} NBD, and included residues in an extended loop of IIB ²⁴. Specific residues in DnaK_{Tth} that could potentially form H-bonds with ClpB M-domain residues were identified (R55 and K285 in subdomain IB and the IIB loop respectively; homologous to E. coli R56 and T291) and were in a region previously shown by x-ray crystallography to interact with GrpE (Fig. 1B and D)^{12, 24}. Additional functional and NMR based experiments suggested that GrpE and ClpB compete for binding to the DnaK nucleotide-binding domain ²⁴. In another report, the N-terminal domain of human Hsp70 (HSPA1A) was shown to interact with the M-domain of Saccharomyces cerevisiae Hsp104, but the N-terminal domain alone was insufficient to stimulate disaggregation activity in collaboration with Hsp104²⁷.

A recent study by Reidy et al. showed that *E. coli* ClpB, DnaK and GrpE support prion propagation and thermotolerance in *S. cerevisiae*, when expressed from plasmids, thus replacing the yeast chaperones, Hsp104 and Ssa1 for these activities ²⁸. These results suggested that ClpB is able to replace Hsp104 for most cellular activities as long as the species-specific Hsp70 protein, DnaK, is present. Surprisingly, GrpE, which is known to function with DnaK but not Ssa1 ^{29, 30}, was also required for these reactions pointing to an essential role for GrpE during protein disaggregation and reactivation with ClpB and DnaK. In contrast *E. coli* DnaJ, which can function with Ssa1 and other Hsp70s ^{21, 23}, was not essential ²⁸.

Here, we examined collaboration between the *E. coli* chaperones and co-chaperones involved in protein disaggregation. We identified a region of $DnaK_{Ec}$ that is important for interaction and collaboration with $ClpB_{Ec}$. This region includes residues in the IB and IIB subdomains of the $DnaK_{Ec}$ NBD, including residues in the GrpE binding site. Additionally, modeling studies support a mutually exclusive GrpE and $ClpB_{Ec}$ binding site on $DnaK_{Ec}$ and indicate that DnaK interacts with two adjacent ClpB protomers within a hexamer.

Results

Recent in vitro studies from Rosenzweig et al. ²⁴, using *T. thermophilus* chaperones showed that ClpB_{Tth} interacts with residues in the GrpE binding site of DnaK_{Tth}. In addition the data suggested that GrpE inhibits disaggregation by ClpB, DnaK and DnaJ by competing with ClpB for binding to the DnaK nucleotide-binding domain (NBD) ²⁴. From these results they proposed that GrpE is not directly involved in protein disaggregation by DnaK, DnaJ and ClpB ²⁴. These results are in apparent contradiction with the studies of Reidy et al., showing that *E. coli* GrpE, as well as DnaK and ClpB, is required for prion propagation and thermotolerance in yeast cells deleted for Hsp104 ²⁸. Because GrpE is specific for DnaK_{Ec} and does not act with Ssa1 ^{29, 30}, this work implied that GrpE is directly involved in disaggregation and prion propagation with ClpB and DnaK. Therefore, we investigated whether the region in the NBD of DnaK_{Ec} that is involved in GrpE is involved in protein disaggregation with ClpB_{Ec} in protein remodeling and whether GrpE is involved in protein disaggregation with ClpB, DnaK and DnaJ.

Residues of the *E. coli* DnaK NBD subdomain IIB are important for functional collaboration between *E. coli* DnaK and ClpB

We first tested whether residues in the GrpE binding region of the *E. coli* DnaK NBD are involved in functional interaction and collaboration with *E. coli* ClpB, as they are in *T. thermophilus* DnaK. Several variants were constructed containing amino acid substitutions in the NBD of DnaK_{Ec} in residues shown by X-ray crystallography to be involved in GrpE binding (Table 1 and Fig. 1B and D) ¹². Some were single substitutions and others were double or triple substitutions, denoted by an (*) after the first residue (Table 1). The DnaK mutants were then tested for their ability to reactivate heat-denatured GFP (hdGFP) with DnaJ and GrpE or with ClpB, DnaJ and GrpE. As previously seen, wild type DnaK with DnaJ and GrpE reactivated hdGFP slowly; the addition of ClpB stimulated the rate of reactivation by ~3-fold (Fig. 2A and Supplemental Fig. S1A) ³¹. When DnaK mutants with

substitutions in subdomain IIB, including DnaK_{L257A}, DnaK_{R261C} and DnaK_{N282A*}, were tested, we observed that they were able to reactivate hdGFP, although the rates of reactivation were slower than for wild type (Fig. 2A and B and Supplemental Fig. S1A). Importantly, reactivation was not appreciably stimulated by the addition of ClpB, suggesting that these residues are important for the collaboration between DnaK and ClpB. In contrast, DnaK mutants with substitutions in the subdomain IIB loop, DnaKA288C and DnaKA290C, had activity similar to DnaK_{wt} either with DnaJ and GrpE or with ClpB, DnaJ and GrpE, indicating these substitutions do not affect the collaboration with ClpB for the reactivation of hdGFP (Fig. 2A and Supplemental Fig. S1A). DnaK with substitutions in residues in subdomain IB DnaKL49A, DnaKR56A and DnaKQ57A*, were partially defective in protein reactivation in collaboration with DnaJ and GrpE, but ClpB stimulated the rate of disaggregation between ~5 and 15-fold (Fig. 2C and Supplemental Fig. S1A). The results suggest that the subdomain IB residues that we characterized are important for DnaK, DnaJ and GrpE reactivation of hdGFP. However, they are not important for reactivation of hdGFP in collaboration with ClpB. In additional experiments, DnaK mutants with substitutions in other regions of the DnaK NBD were tested for the ability to reactivate hdGFP (Table 1 and Supplemental Fig. S1B and S1C). Many of these DnaK mutants were defective in hdGFP reactivation with DnaJ and GrpE, however ClpB stimulated hdGFP reactivation by all mutants at least ~3-fold (Supplemental Fig. S1B and S1C). Together these results show that residues in the GrpE binding region of E. coli DnaK are important for DnaK collaboration with E. coli ClpB.

Based on our previous observation that the DnaK system and ClpB act synergistically to hydrolyze ATP in the presence of substrate ³¹, we next tested if the DnaK mutants were defective in this activity. We monitored ATPase activity by the mutant DnaK proteins both with and without ClpB in the presence of DnaJ, GrpE and heat-denatured malate dehydrogenase (hdMDH). As previously seen, the ATPase activity of ClpB in combination with the DnaK system in the presence of hdMDH was ~2.5-fold higher than the additive rates of the DnaK system and ClpB separately in the presence of hdMDH (Fig. 3A)³¹. In contrast, when the subdomain IIB mutants, DnaK_{L257A}, DnaK_{R261C} and DnaK_{N282A*}, were incubated with ClpB, DnaJ, GrpE and hdMDH, ATP hydrolysis rates were not stimulated above the additive rates of the DnaK mutants with cochaperones and ClpB separately (Fig. 3A). The ATPase activity exhibited by subdomain IB mutant, DnaK_{R56A}, with ClpB, DnaJ, GrpE and hdMDH was approximately additive and similar to the subdomain IIB mutants, suggesting a defect in collaboration between ClpB and this mutant (Fig. 3B). The ATPase activity of other subdomain IB mutants, DnaK_{Q57A*} and DnaK_{L49A} and subdomain IIB loop mutants, DnaKA288C and DnaKA290C was stimulated ~1.6-2.3-fold more than additive in the presence of ClpB, DnaJ, GrpE and hdMDH, however, the stimulation was less than the ~2.5-fold stimulation observed for DnaK_{wt} (Fig. 3B). For other E. coli DnaK mutant proteins with substitutions in the NBD, DnaK_{Y145A*} and DnaK_{N187A*}, the ATPase activity in the presence of DnaJ, GrpE, ClpB and hdMDH was between ~1.7-2.1-fold more than additive, again similar to DnaKwt (Supplemental Fig. S1D). Together, these results show that residues in DnaK_{Ec} subdomains IB and IIB are important for functional collaboration between ClpB and DnaK.

E. coli DnaK variants with substitutions in subdomains IB and IIB of the NBD are defective for in vivo and in vitro interactions with CIpB

To determine if variants that were defective in functional collaboration with ClpB were defective in intermolecular interaction with ClpB, we tested our mutants using a bacterial two-hybrid assay ³². We had previously used this assay to demonstrate an interaction between DnaK and ClpB in vivo ²⁵. DnaK wild type or mutant was fused to the C-terminus of one fragment of Bordetella pertussis adenylate cyclase, T25, while ClpB was fused to the C-terminus of the other adenylate cyclase fragment, T18. If ClpB and the DnaK mutant being tested interact, the T18 and T25 fragments will form an active adenylate cyclase and a cAMP dependent reporter gene, β -galactosidase, is expressed. As previously observed, coexpression of T18-ClpB and T25-DnaKwt resulted in higher levels of β-galactosidase activity than coexpression of T18-ClpB and the T25 vector, suggesting that ClpB and DnaK interact in vivo (Fig. 4A)²⁵. When the T25-subdomain IIB mutant fusions, T25-DnaK_{L257A}, T25-DnaK_{R261C} or T25-DnaK_{N282A*}, were each coexpressed with T18-ClpB, we observed less β -galactosidase activity than seen with T25-DnaK_{wt}, indicating that the mutants were partially defective in ClpB interaction in vivo (Fig. 4A). The subdomain IB mutants, DnaK_{R56A} and DnaK_{O57A*}, were also partially defective in ClpB interaction in vivo (Fig. 4A). In control experiments all of the mutant proteins were produced at levels similar to T25-DnaK_{wt} (Supplemental Fig. S2).

We tested DnaK mutants for interaction with ClpB in vitro by measuring the fluorescence anisotropy signal of pyrene-labeled DnaK in the presence and absence of ClpB. To do this, a K106C substitution was introduced into DnaK_{wt} and the DnaK mutants and then the purified proteins were labeled with pyrene-maleimide, referred to as DnaK_{py-wt}, DnaK_{py-L257A}, DnaK_{py-N282A*}, DnaK_{py-R56A}, and DnaK_{py-Q57A*}. A ClpB mutant that contains mutations in each NBD (E279A and E678A) ³³, ClpB_{trap}, was used to promote the ClpB ATP bound conformation and stabilize the DnaK-ClpB interaction. We observed that the addition of ClpB_{trap} and ATP to DnaK_{py-Wt} stimulated the anisotropy signal ~2.2-fold above the DnaK_{py-wt} signal alone, indicating an interaction between the two proteins (Fig. 4B). The anisotropy signals of DnaK_{py-L257A}, DnaK_{py-N282A*}, DnaK_{py-R56A} and DnaK_{py-Q57A*} also increased upon addition of ClpB, however the increase was only ~1.2–1.3-fold above the signal for the DnaK mutant alone (Fig. 4B). Taken together, the results suggest that the decrease in the functional collaboration in ATPase and protein remodeling activities between *E. coli* ClpB and the DnaK mutants in subdomain IB and IIB is likely caused by a weaker interaction between the ClpB M-domain and DnaK.

GrpE competes with ClpB for a binding site on DnaK

Since the region on *E. coli* DnaK involved in collaboration and interaction with ClpB (Fig. 2, 3 and 4A and B) is also involved in interactions with GrpE ¹², we tested whether GrpE competes with ClpB for DnaK interaction in vitro. Fluorescence anisotropy was carried out using pyrene labeled wild type DnaK as above. We observed that the fluorescence signal seen by the combination of DnaK_{py-wt}, ClpB_{trap} and ATP decreased by ~33% with the addition of GrpE at a concentration equimolar to ClpB (Fig. 4C). These data suggest that GrpE interferes with the interaction between ClpB and DnaK. As a control, excess unlabeled

 $DnaK_{wt}$ caused a decrease in signal of ~30% compared to $DnaK_{py-wt}$ and $ClpB_{trap}$ alone (Fig. 4C), indicating that unlabeled $DnaK_{wt}$ competes with $DnaK_{py-wt}$ for binding to ClpB.

E. coli GrpE and ClpB can both collaborate with DnaK during the process of protein remodeling

We next wanted to explore the proposal by Rosenzweig et al. that GrpE does not aid ClpB, DnaK and DnaJ in extracting polypeptides from aggregates, but rather participates in a separate later reaction with DnaK and DnaJ to refold the unfolded polypeptides released from ClpB²⁴. To eliminate the effects of the DnaK system in protein refolding following release from ClpB, we utilized RepA as a substrate. RepA is activated for DNA binding by conversion of inactive dimers to active monomers using the combined activity of ClpB, DnaK and DnaJ³¹. RepA does not require chaperone assisted refolding of monomers following dissociation of dimers into monomers ³⁴. Our lab has also shown that with some conditions DnaK and DnaJ alone activate RepA ³¹ and with other conditions activation requires GrpE in addition to DnaK and DnaJ³⁵. For example, DnaK and DnaJ efficiently remodel RepA dimers into monomers in the absence of GrpE when the concentration of DnaK is ~1.0 μ M (Fig. 5A). However, at lower concentrations of DnaK (~0.5 μ M), GrpE is required for RepA remodeling (Fig. 5A). We observed that RepA activation was stimulated by either a low (0.03 μ M) or a high concentration of GrpE (2 μ M), but the lower concentration was more effective (Fig. 5A). Moreover, as we increased the concentration of GrpE in the presence of DnaK (0.4 μ M) and DnaJ, RepA activation was stimulated and then inhibited (Fig. 5B). RepA activation decreased at ratios of 1:0.5 or lower DnaK to GrpE. Previously, inhibition of DnaK chaperone activity by excess GrpE was observed ^{36–39} and it was suggested that a balance between ATP binding and ATP hydrolysis must be maintained for optimal chaperone activity. Thus, the presence of excess GrpE likely shifts the balance towards ATP-bound DnaK, which has low affinity for substrate, and would lead to less RepA being bound and activated by DnaK.

We next investigated whether GrpE stimulated or inhibited RepA activation by the combination of DnaK, DnaJ and ClpB. A lower concentration of DnaK_{wt} (0.2 µM) was sufficient for RepA activation in the presence of ClpB compared to in the absence (Fig. 5C) ³¹. The addition of GrpE (0.03 μ M) to the ClpB, DnaK and DnaJ reaction did not alter the requirement for DnaK, neither stimulating nor inhibiting the reaction (Fig. 5C). However, at a higher GrpE concentration (2 µM), a higher concentration of DnaK_{wt} (0.5 µM) was required for RepA activation, suggesting that GrpE may interfere with the collaboration between ClpB and DnaKwt for the monomerization of RepA (Fig. 5C). To better understand the effect of GrpE on the activation of RepA by DnaK, DnaJ and ClpB, we varied the GrpE concentration in the reaction (Fig. 5D). Addition of GrpE up to $\sim 0.05 \,\mu M$ slightly stimulated RepA activation by DnaK, DnaJ and ClpB; at higher GrpE concentrations, chaperone activity was inhibited (Fig. 5D). Similar to our results in the absence of ClpB (Fig. 5B), RepA activation decreased at ratios of 1:0.5 or lower DnaK (0.1 μ M) to GrpE in the presence of ClpB. These results indicate that GrpE is essential for the activation of RepA at low concentrations of DnaK in the absence of ClpB. But when ClpB is present, GrpE is not required for RepA monomerization and GrpE inhibits the reaction at high concentrations. GrpE may inhibit RepA activation by ClpB, DnaK and DnaJ by

competing with ClpB for binding to DnaK (Fig. 5D). However, the decrease in RepA activation observed for the DnaK and DnaJ reaction with high GrpE concentrations makes it difficult to interpret the results (Fig. 5B).

The effect of GrpE on the remodeling of another substrate, hdGFP, by the DnaK system and ClpB was also tested. Rosenzweig et al., used this substrate and found that GrpE inhibited the disaggregation of GFP when a 10:2:1 molar ratio of *T. thermophilus* DnaK:DnaJ:GrpE was used in the presence of ClpB_{Tth}²⁴. We monitored hdGFP reactivation by *E. coli* DnaK and DnaJ or DnaK, DnaJ and ClpB in the absence or presence of GrpE. In the presence of a 10:2:1 ratio of DnaK:DnaJ:GrpE, we observed that the initial rate of hdGFP reactivation was two-fold higher in the presence than in the absence of GrpE (Fig. 5E). Moreover, the 2-fold GrpE stimulation was also seen when ClpB was present (Fig. 5E). A similar stimulation by GrpE was observed using a molar ratio of 10:2:10 DnaK:DnaJ:GrpE either without or with ClpB (Fig. 5E). These results suggest that GrpE aids in the reactivation of hdGFP by DnaK and DnaJ, and under the conditions tested it does not interfere with the stimulation observed upon addition of ClpB. Taken together, the data suggest that the roles ClpB and GrpE play during collaboration with DnaK may be overlapping, but they are not identical, despite sharing a binding site on DnaK.

Subdomains IB and IIB of eukaryotic Hsp70 are important for collaboration with Hsp104

Since yeast Hsp104 and Hsp70, like E. coli ClpB and DnaK, interact in a species-specific manner ^{21, 25–27, 40, 41}, we tested if the same region of yeast Hsp70, Ssa1, was important for collaboration with Hsp104. We constructed mutants in subdomains IB, IIB and the IIB loop of the Ssa1 NBD (Table 2). The Ssa1 mutants were tested for the ability to collaborate with Hsp104 in reactivating heat-denatured luciferase (hdLuc) (Fig. 6). Ssa1wt reactivated hdLuc in combination with yeast Hsp40, Ydj1, and the addition of Hsp104 stimulated the rate of the reaction ~2-fold, as previously observed (Fig. 6A) ^{25, 42}. Ssa1_{S283A} reactivated hdLuc as well as Ssa1wt and was further stimulated by Hsp104 (Fig. 6A). Ssa1D282A also reactivated hdLuc similarly to Ssa1_{wt}, but the addition of Hsp104 did not stimulate the reaction, suggesting that this residue is likely important for collaboration with Hsp104 (Fig. 6A). At the same concentration as Ssa1wt, Ssa1R255A, Ssa1R259A, Ssa1G287A and Ssa1D289A had limited activity with Ydj1. When these mutant proteins were added at a 3-fold higher concentration, reactivation of hdLuc was observed, but Hsp104 did not stimulate reactivation further (Fig. 6B). The subdomain IB mutants, Ssa1_{L48A} and Ssa1_{O56A}, had the ability to act with Ydj1 similar to Ssa1wt (Fig. 6C). However, Ssa1L48A had a slight defect in collaboration with Hsp104, and Ssa1_{Q56A} was unable to collaborate with Hsp104 (Fig. 6C). Together these results suggest that the region on DnaK and Ssa1 that interacts with ClpB/ Hsp104 is similar, but not identical (Fig. 6A–C).

A substitution mutant in subdomain IIB of human Hsp70, $hHsp70_{R262A}$ (homologous to $DnaK_{R261}$ and $Ssa1_{R259}$), was also tested for the ability to collaborate with Hsp104 and Ydj1 for the reactivation of hdLuc. This mutant was defective in collaboration with Hsp104 in agreement with the Ssa1 and DnaK results (Supplemental Fig. S3). Together, these results suggest that NBD subdomains IB and IIB of eukaryotic homologues of DnaK are important

for specific collaboration with Hsp104 in protein reactivation, implying that this region of Hsp70 is conserved among species as a site of ClpB/Hsp104 interaction.

Computational modeling of the DnaK-ClpB complex indicates multivalent binding of DnaK to ClpB

Our experimental data suggest that the collaboration between ClpB and DnaK in reactivation reactions is inhibited by high concentrations of GrpE. Moreover, the mutants that we found to be defective in interaction with ClpB were in residues known to interact directly with GrpE. These results imply that ClpB and GrpE interact with DnaK in a mutually exclusive manner. To gain a better understanding of the interaction of DnaK with ClpB, we employed a computational approach. We utilized the available solution NMR structure of E. coli DnaK in the ADP bound conformation ¹⁸ and we constructed both trimer and hexamer models of E. coli ClpB. The trimer model was based on the crystal structure of trimeric T. thermophilus ClpB, which presented three distinct subunit conformations ⁴³. In the ClpB_{Ec} hexamer model we accounted for these distinct subunit conformations and addressed asymmetry by satisfying restraints provided by the recently reported asymmetric EM map of E. coli ClpB (EMD-2558)⁴⁷ as described in Methods. Binding models of the DnaK-ClpB complex were obtained by rigid body docking ⁴⁴ of DnaK with the ClpB trimer and hexamer models (see Methods)⁴⁴. The resulting decoys were ranked based on cluster size and then compared with experimental data. The top ranking DnaK-ClpB decoy generated using the ClpB hexamer model is discussed below. The decoy generated using the DnaK-ClpB trimer model was very similar and is described in Supplemental Information (Supplemental Table S2 and Supplemental Figure S4).

Analysis of the top docking DnaK-ClpB hexamer decoy indicated a binding interface on DnaK involving subdomains IB and IIB of the NBD. The decoy further showed DnaK interacting with the M-domain of one ClpB protomer and with the M-domain and NBD-1 of an adjacent ClpB protomer (Fig. 7). The majority of the DnaK residues shown to be involved in the collaboration between DnaK and ClpB by protein remodeling assays were in contact with the ClpB M-domains (Table 3 and Supplemental Table S1). These include subdomain IB residues R56 and V59 and subdomain IIB residues R261, P284 and Y285. DnaK interacted with one ClpB subunit (chain E) in motif 2 of the M-domain (Fig. 1C) between amino acid 490 and 518, forming 14 contacts within a cutoff of 6 Å (Fig. 7 and Supplemental Table S1). DnaK made 16 additional contacts with motif 1 of the M-domain of the adjacent ClpB protomer (chain F) between amino acids 423 and 456 (Fig. 7 and Supplemental Table S1). In addition, there were seven contacts between DnaK and the NBD-1 of ClpB (chain E) (Fig. 7 and Supplemental Table S1). An interaction between DnaK and NBD-1 of ClpB has not been experimentally identified, and further studies are necessary to corroborate these predictions. The model corresponds well with recent studies, which show that residues between amino acids 435-448 in motif 1 of the M-domain of Hsp104 (corresponding to residues 433–444 of $ClpB_{Fc}$) are involved in collaboration and interaction with Hsp70^{25–27, 40, 41, 45}. Additionally, homologous substitution mutants in *E*. coli ClpB are defective for collaboration with DnaK in protein remodeling ^{25–27, 40, 41}. Altogether the protein-protein interactions identified in the model of the DnaK-ClpB hexamer complex support the functional and direct interactions identified in biochemical

studies. Furthermore, they suggest a mechanism for collaboration between DnaK and ClpB for protein disaggregation.

Next, we compared the DnaK-ClpB binding interface identified in our modeling study to the DnaK-GrpE binding interface as determined by crystal structure ¹² (see Methods). We used the DnaK-GrpE crystal structure to evaluate the change in SASA of the experimentally identified DnaK residues that are important for ClpB binding, including DnaK subdomain IB and IIB residues R56, V59, T60, L257, R261, N282, P284, and Y285 (Table 3). The SASA of these residues in DnaK-GrpE complexes was greatly reduced compared to DnaK alone, showing that these residues are buried (R56, L257, R261, and P284) or partially buried (V59, T60, N282 and Y285) when DnaK interacts with GrpE (Table 3 and Supplemental Fig. S5). Additionally, each of these DnaK residues made 1–3 contacts with GrpE. The results of this analysis are consistent with the experimental results suggesting that ClpB and GrpE compete for the same interaction site on DnaK, and bind in a mutually exclusive fashion.

Collectively, the results presented here indicate that the interaction between DnaK and ClpB comprises one step in the protein disaggregation pathway, and suggest that GrpE is not present on ClpB-bound DnaK. However, they do not rule out a role for GrpE in the disaggregation process.

Discussion

Our results show that residues within subdomains IB and IIB of the NBD of *E. coli* DnaK are important for interaction and collaboration with *E. coli* ClpB, consistent with recent results using *T. thermophilus* homologues ²⁴. Since many of the residues identified as part of the ClpB binding region of DnaK_{Ec} and DnaK_{Tth} were shown previously to interact with GrpE ^{12, 24}, the observations presented here and recently ²⁴ suggest that ClpB and GrpE of *E. coli* and *T. thermophilus* utilize the same DnaK binding region in a mutually exclusive fashion. We also found that yeast Hsp104 interacts with subdomain IB and IIB of Ssa1 through residues homologous to those in DnaK that interact with ClpB. However, it remains to be explored whether yeast NEF and Hsp104 utilize a single binding site on Ssa1.

The results further indicate that there are some differences in the interaction and collaboration between DnaK and ClpB in different species. For example, we observed that collaboration between DnaK_{Ec} and ClpB_{Ec} for hdGFP reactivation was unperturbed at a 1:1 molar ratio of DnaK_{Ec} to GrpE_{Ec} with or without ClpB (Fig. 5E). In contrast, GrpE_{Tth} inhibited the collaboration between ClpB_{Tth} and DnaK_{Tth} for hdGFP disaggregation, even at substoichiometric levels (10:1 DnaK_{Tth} to GrpE_{Tth})²⁴. Some, but not all of the DnaK_{Tth} residues shown by NMR structural analysis to interact with ClpB_{Tth}²⁴ were identified as DnaK_{Ec} residues involved in collaboration with ClpB_{Ec}. This could explain the differences observed for GrpE competition with ClpB for binding on DnaK_{Ec} versus what has been observed for the *T. thermophilus* chaperones ²⁴. The loop in subdomain IIB appears to be important for interaction with ClpB_{Tth} ²⁴, but substitutions in two loop residues in DnaK_{Ec} did not alter either intrinsic or collaborative activity of DnaK_{Ec} with ClpB_{Ec}. Interestingly, substitutions in the IIB loop of Ssa1 cause major defects in chaperone activity as well as a

loss in collaboration with Hsp104. The DnaK_{Tth} loop is two amino acids longer while the Ssa1 loop is four residues shorter than the ~12 residue DnaK_{Ec} loop, possibly accounting for differences in the collaboration in protein reactivation with the corresponding disaggregase.

Our results from the characterization of DnaK mutants are in agreement with several recently published studies: 1) an NMR structural study of the DnaK_{Tth}-ClpB_{Tth} interaction ²⁴, 2) an HD exchange study showing that some residues in the ClpB M-domain helix 2 of motif 1 (Fig. 1C) are solvent exposed in hexameric ClpB_{Ec} ⁴⁶, and 3) crosslinking studies demonstrating that residues in M-domain helices 2 and 3 of motif 2 (Fig. 1C) directly interact with DnaK_{Ec} ²⁶. Our modeling study of the DnaK-ClpB hexamer (shown in Fig. 7) predicts that DnaK interacts simultaneously with the M-domains from two adjacent ClpB protomers, a result with potential functional implications. In this model residues in M-domain helices 2, 3 and 4 (motif 2) of the neighboring ClpB protomer (chain E) contribute to the binding of a single DnaK.

The structural predictions of our model and that of Rosenzweig et al. ²⁴ differ. The Rosenzweig et al. model was generated using the NBD fragment of DnaK and a fragment of a ClpB protomer containing the NBD-1 and M-domain, but lacking the N-domain and NBD-2 ²⁴. Due to the use of the truncated structures and the absence of the oligomeric assembly of ClpB, this model could only predict complexes that involve binding sites from a single ClpB subunit. Additionally, possible steric clashes may occur with structural overlapping of the docked DnaK-ClpB fragments with full-length DnaK and oligomeric ClpB. In contrast, the model presented here used full-length DnaK and the ClpB hexamer model generated in this study (Fig. 7). Although our modeling did not preclude interactions of DnaK with a single ClpB protomer as proposed by the Rosenzweig et al. study ²⁴, our results suggested binding of DnaK to two neighboring ClpB protomers as the most favorable interaction for complex formation.

Our modeling data indicating multivalent binding of DnaK to the ClpB hexamer through the M-domains of adjacent ClpB protomers is consistent with the recent EM study of Carroni et al. ⁴⁷. The EM data suggests that the ClpB M-domains adopt various positions in a single ClpB hexamer ⁴⁷, as does the crystal structure of the T. thermophilus ClpB trimer ⁴³. Studies of a DnaK-independent mutant with a substitution in motif 2 showed that the M-domains were tilted upward, while the M-domains of a motif 1 mutant deficient in DnaK interaction were horizontal. The results suggest that when adjacent M-domains are horizontal, the Mdomains form head-to-tail contacts preventing DnaK interaction and repressing ClpB activity. The model presented by Carroni et al. proposes that when an M-domain transiently tilts upward, contacts between it and the adjacent M-domains are broken, DnaK binds to motif 2 of the M-domain and ClpB disaggregase activity is activated ⁴⁷. In addition, our model also predicts that DnaK contacts two ClpB protomers simultaneously. While the two models differ in how ClpB interacts with DnaK, both are consistent with the idea that the binding of one or more DnaK to a ClpB hexamer promotes a functionally active conformation of ClpB, poised for protein disaggregation. We expect that future studies will continue to reveal information regarding the DnaK-ClpB hexamer interaction and its functional implications.

In summary, the complexity surrounding protein disaggregation and refolding is apparent. Not only are DnaK and ClpB or Ssa1 and Hsp104 major players in this process, but also cochaperones, specifically the nucleotide-exchange factors, add another layer of regulation to the process. Whether the competition between ClpB and GrpE for binding to DnaK plays a role in protein disaggregation or other DnaK processes in the cell has yet to be addressed.

Materials and Methods

Strains and Plasmids

Plasmids p18link-ClpB and p25link-DnaK (Supplemental Table S3) were transformed into BTH101 cells for expression ^{25, 48}. The plasmids for expression of DnaK mutants (Table 1) were constructed using the QuikChange site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA) and plasmid pET24b-dnak or p25link-DnaK (Supplemental Table S3) as template. Plasmid for the expression of the human Hsp70 (HSPA1A) was cloned into pET-23c and was a gift from Len Neckers (NIH). The plasmid was transformed into BL21(DE3)pLysS cells. Human Hsp70 mutants and yeast Ssa1 mutants (Table 2) were constructed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and plasmids pET23c-*HSPA1A* (Supplemental Table S3) and pET24b-*SSA1* respectively as templates.

Proteins

P1 RepA ⁴⁹, GFP ⁵⁰, wild type and mutant ClpB ⁵¹, Hsp104 ²⁵, wild type and mutant DnaK ³⁵, DnaJ ³⁵, GrpE ³⁵, wild type and mutant human Hsp70 ²⁵, Ydj1 ⁵² and [³H]*ori*P1 DNA ⁴⁹ (4475 cpm/fmol) were prepared essentially as described. Wild type and mutant Ssa1 were purified as previously described for DnaK ³⁵ with some modifications. MDH was purchased from Roche (Indianapolis, IN). Luciferase was purchased from Promega (Madison, WI). Protein concentrations given are for monomeric DnaK, Ssa1, human Hsp70, luciferase and GFP, dimeric DnaJ, Ydj1, GrpE and MDH, and hexameric ClpB and Hsp104.

ATPase activity

Reaction mixtures (50 μ L) contained HKE buffer [25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA], 0.005% Triton X-100 (vol/vol), 2 mM DTT, 4 mM ATP, 0.1 μ Ci of [γ -³³P]ATP (>3000 Ci/mM; GE Healthcare), 10 mM MgCl₂, 1.3 μ M DnaK wild-type or mutant, 0.25 μ M DnaJ, 0.13 μ M GrpE, 0.4 μ M ClpB, 0.6 μ M heat-denatured MDH (heated at 47 °C for 33 min in Buffer B [50 mM Tris, pH 7.5, 150 mM KCl, 20 mM MgCl₂] at 2 μ M) as indicated. Reactions were initiated by the addition of ATP, incubated 60 min at 25°C and analyzed as described ⁵³.

GFP disaggregation

GFP reactivation assays (100 μ L) contained HKE buffer, 5 mM DTT, 4 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 0.3 μ g/ml creatine kinase), 20 mM MgCl₂, 10 μ L heat-aggregated GFP (heated 10 min at 80 °C at 4.5 μ M) and, where indicated, 1.4 μ M wild-type or mutant DnaK, 0.25 μ M DnaJ, 0.13 μ M or 1.3 μ M GrpE and 0.25 μ M ClpB. Reactivation was monitored over time at 23 °C using a Tecan Infinite M200Pro plate reader. Excitation and emission wavelengths were 395 nm and 510 nm,

respectively. Refolding rates were determined from the initial linear increase in fluorescence intensities of GFP.

RepA activation

Reaction mixtures ($20 \ \mu$ L) contained HKE buffer, 2 mM DTT, 4 mM ATP, 10 mM MgCl₂, 50 µg/ml BSA, 0.005% Triton X-100 (vol/vol) and 7 nM RepA. For DnaK titrations, DnaK concentration varied between 0 and 2 µM protein as indicated, with 15 nM DnaJ, 180 nM ClpB as indicated, and either 30 nM or 2 µM GrpE as indicated. For GrpE titrations, GrpE concentration varied between 0 and 2 µM protein as indicated, with 15 nM DnaJ, 100 nM or 400 nM DnaK as indicated, and 20 nM ClpB as indicated. For MgCl₂ titrations, reaction mixtures contained HK buffer [25 mM Hepes, pH 7.5, 50 mM KCl], 0.01 to 15 mM MgCl₂, 0.3 mM EDTA, 0.6 µM DnaK, 25 nM DnaJ, 30 nM GrpE and 180 nM ClpB as indicated. After 10 min at 23 °C the reactions were chilled to 0 °C. Calf thymus DNA (1 µg) and 13 fmol of [³H]*ori*P1 plasmid DNA were added. After 5 min at 0 °C, the mixtures were filtered through nitrocellulose filters and retained radioactivity was measured.

Heat-denatured luciferase reactivation

Luciferase (40 nM) was denatured in HKE buffer (55 μ l) supplemented with 0.05 mg/ml BSA, 2 mM DTT and 10 mM MgCl₂ for 7 min at 45 °C followed by 1 min at 4 °C in a thermocycler. Denatured luciferase was added to reaction mixtures (final total volume of 75 μ L) containing HKE buffer, 3 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 0.3 μ g/ml creatine kinase), 15 mM MgCl₂, 2 μ M Ssa1 wild type or mutants as indicated, 1 μ M Ydj1, and 0.4 μ M Hsp104 as indicated. Reactions were incubated at 23 °C for 30 min and aliquots (5 μ L) were removed at the times indicated. Luciferase activity was determined at 23 °C by injecting 120 μ L of 25 mM Hepes, pH 7.5, 0.5 mM ATP 10 mM MgCl₂ and 200 μ M luciferin (Roche) and measuring light output in a Tecan Infinite M200Pro plate reader. Reactivation was determined compared to a non-denatured luciferase control.

Fluorescence anisotropy

Reaction mixtures (150 µL) contained HKE buffer with 0.5 mM TCEP-HCl from Thermo Scientific (Rockford, IL), 10 mM MgCl₂, 4 mM ATP, 0.075 µM DnaK_{K106C} single or double mutant labeled with N-(1-pyrene)maleimide as per included instructions from Invitrogen (Carlsbad, CA) (degree of labeling was between 0.1 and 0.5 for all mutants) and 5 µM ClpB_{E279A,E678A}. 5 µM DnaK wild type or 5 µM GrpE were added as indicated. Fluorescence anisotropy measurements were performed on a Perkin Elmer LS55 spectrofluorimeter with excitation and emission wavelengths set at 338 nm and 380 nm, respectively with 10 nm slits, and with polarizers in place. Data was collected for 15 min at a frequency of ~1 point every 10 sec and the average anisotropy signal between 400 to 900 sec was determined.

Bacterial two-hybrid system

A bacterial two-hybrid system utilizing *B. pertussis* adenylate cyclase was used as previously described ^{25, 32}. The *clpB* coding region was fused in frame to the 3' end of the

T18 fragment (pEB355) and *dnaK* wild type or mutant was fused to the 3' end of the T25 fragment (pEB354) of *B. pertussis* adenylate cyclase (see Supplemental Table S3). Plasmid pEB354 carrying *dnaK* wild type or mutant, p25link-DnaK (see Supplemental Table S3), was co-transformed with p18link-ClpB into BTH101 (*E. coli cya*) cells. Cells were plated on LB plates containing ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and incubated at 30°C. Single colonies were selected and used to inoculate LB selective media and cultures grown at 30 °C. β -galactosidase reporter activity was measured as previously described ⁵⁴.

Modeling of protein-protein interactions

A. Modeling of *E. coli* ClpB (ClpB_{Ec}) monomeric and oligomeric structures— Full-length monomeric structures of ClpB_{Ec} subunits were obtained through the threading approach available in the I-TASSER structure prediction software (http:// zhanglab.ccmb.med.umich.edu/I-TASSER/)⁵⁵. Templates were chosen from the *T. thermophilus* ClpB (ClpB_{Tth}) counterpart (Protein Data Bank (PDB) entry 1QVR) ⁴³, which has 56% sequence similarity with ClpB_{Ec}. Although the functionally active state of ClpB is a hexamer, the only well-resolved crystal structure of oligomeric ClpB is a trimer. While all ClpB_{Tth} subunits have the same sequence, each protomer possesses a distinct orientation of the N-terminal domain (residues 1-157) and the M-domain (residues 401–512). This structural heterogeneity is reflected in relatively small (~2–3Å) root-mean-square deviations (RMSDs) between subunit pairs of ClpB_{Tth} N and large RMSDs of ~2.5–10.5Å between wild-type subunit pairs. To account for the intra-ring asymmetry, our first step was to obtain models of ClpB_{Ec} using individual ClpB_{Tth} subunits (A, B, and C) as templates.

The $ClpB_{Fc}$ hexamer model was generated to satisfy restraints provided by the asymmetric electron microscopy map of E. coli ClpB (EMD-2558) 47. In addition, we maintained consistency with the subunit ordering found in the ClpB_{Tth} trimer. The map restraints were generated as follows: Fragments of NBD-1-M (residues 161-550) from I-TASSER generated ClpB_{Ec} subunits A, B, and C were fitted into the density map using Chimera ⁵⁶. Dimers of ClpB_{Ec} NBD-2 were then fit into the electron density map. The NBD-2 dimers were built using a structural alignment in PyMol 57 to minimize the RMSD between NBD-2 fragments of individual ClpB subunits (residues 601-858) and the ClpX ring (PDB ID 3HWS) ⁵⁸. Next, full-length individual subunits generated by I-TASSER were fit into the density map, with subunit sequence ABCABC (counterclockwise orientation viewed along the pore axis from NBD-1) that preserved the asymmetric subunit ordering found in the ClpB_{Th} trimer. This structure was then minimized to satisfy map restraints by imposing best-fit positional restraints for NBD-1, NBD-2 and M-domain atoms. To this end, we performed 20000 steps of steepest descent and 1000 steps of adapted basis Newton-Raphson minimization using CHARMM ⁵⁹. Overall, our final model illustrates the assembly of fulllength ClpB_{Ec} subunits into a hexamer structure compatible with the asymmetric map restraints.

B. Modeling full-length ADP-bound DnaK and the DnaK/GrpE complex—The crystal structure of the DnaK/GrpE complex (PDB: 1DKG)¹² is not fully resolved, lacking linker and peptide-binding domains of DnaK (residues 384-638). We modeled missing DnaK amino acids 384-605 by using the well-resolved isolated ADP-bound DnaK structure

(PDB:2KHO) ¹⁸. To this end, we first used the CHARMM ⁵⁹ molecular modeling program to build missing amino acids 1-3 and 604-605, as well as hydrogen atoms in the 2KHO structure. The missing regions in the DnaK/GrpE complex were then modeled by superimposing the isolated ADP-bound structure of DnaK onto the apo-DnaK in the DnaK/GrpE complex. Next, we minimized the RMSD of the ATPase domain fragment between the two structures (~6.5Å).

C. Protein-Protein docking and selection of decoys of docking complexes by using experimental information on binding interfaces—Protein-protein docking models of the ClpB_{Ec}/DnaK complexes were obtained using the ClusPro server (http:// cluspro.bu.edu/login.php) 44, 60-62. Our approach incorporated experimental data, which showed that a subset of residues within the M-domains of ClpB (residues 409-530) and subdomains IB and IIB of DnaK (residues 38-119 and 228-310 respectively) participate in binding between these complexes. In ClusPro, all of the residues in these regions were defined as attractive restraints. Although these favorable interactions bias the binding towards these regions of ClpB and DnaK complex, they do not preclude binding of other regions. ClusPro pre-processing of the isolated ClpB and DnaK structures included removal of steric clashes by using 300 steps of minimization of the van der Waals energy with fixed backbone restraints using CHARMM ⁵⁹. To remove remaining steric clashes within the docking decoys, we performed an additional 1000 steps of adapted basis Newton-Raphson and 1000 steps of steepest descent minimization in the absence of restraints using CHARMM⁵⁹. The top 30 decoys were ranked based on cluster size and the highest-ranking decoy was chosen. The top 30 decoys from docking were analyzed for protein interaction sites using the SPPIDER webserver (http://sppider.cchmc.org/) ⁶³. This server implements a neural network to determine the probability of forming binding interfaces, while accounting for structural and sequential features of the proteins. Using these predicted binding sites, the models were then matched for overlaps with experimentally identified residues on ClpB and DnaK 12, 24, 26.

Accessible surface area per residue:

We computed the accessible surface area per residue using the formula:

 $r_s = a_s/a_0$

Where a_s^{64} is the accessible surface area of a specific amino acid and a_0^{65} is the reference value given by Gly – X – Gly accessible surface area. Values of ~1 suggest the residue is located on the surface of the protein.

Contacts:

Contacts were determined by calculating the distances between the center of geometry of residue pairs in DnaK and ClpB or GrpE using a cutoff distance of 6Å (Supplemental Table S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NBD	nucleotide-binding domain
SBD	substrate-binding domain
NEF	nucleotide exchange factor
M-domain	middle domain
N-domain	N-terminal domain

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Highlights

- ClpB and DnaK collaborate in protein disaggregation via specific interactions
- Residues in DnaK subdomains IB and IIB are important for cooperation with ClpB
- Homologous residues in yeast Ssa1 are required for collaboration with Hsp104
- Modeling indicates multivalent binding of DnaK to adjacent ClpB protomers
- ClpB and GrpE both function with DnaK in protein remodeling, but not simultaneously



Fig. 1.

Models of the structure of *E. coli* DnaK and *T. thermophilus* ClpB. **A.** Solution NMR structure of DnaK in the ADP bound conformation (pdb:2KHO) ¹⁸. DnaK is comprised of an N-terminal nucleotide-binding domain (NBD) (blue), containing of four subdomains, IA, IB, IIA and IIB and a substrate-binding domain (SBD) (grey) that contains the β -sheet substrate-binding site and a-helical lid region. **B**. Model of the NBD of DnaK in the ADP bound conformation showing the amino acids identified as interacting with GrpE (cyan) (pdb:2KHO for structure; pdb:1DKG for GrpE binding residues) ^{12, 18}. **C.** Protomer structure of *T. thermophilus* ClpB (PDB ID code 1qvr – chain B) ⁴³, with the N-domain, NBD-1, NBD-2 and M-domain shown. Motif 1 and motif 2 of the M-domain identified (ovals) and the four helices of the M-domain are shown in color: H1 (orange), H2 (purple), H3 (blue) and H4 (green). ATP (black) is shown as a CPK model. **D**. Model of the NBD of DnaK in the ADP bound conformation showing the residues mutated in the present study (pdb:2KHO) ¹⁸. Images in A–D were made using PYMOL (www.pymol.org) ⁵⁷.



Fig. 2.

Substitution mutants in DnaK NBD subdomain IIB are defective for collaboration with ClpB in GFP reactivation. **A–C**. Reactivation of heat-denatured GFP was measured in reactions containing DnaK wild-type or mutant. DnaK wild-type or mutant protein was incubated with DnaJ, GrpE, ClpB and ATP as indicated and the increase in fluorescence intensity was monitored over time as described in methods. Subdomain IIB loop mutants, subdomain IIB mutants and subdomain IB mutants are shown in **A**, **B** and **C**, respectively. In **A–C**, the initial fluorescence was set equal to 0 and a representative experiment is shown (**A–C**) of three or more replicates.



Fig. 3.

DnaK mutants with substitutions in subdomain IB and IIB are defective in synergistic ATPase activity with ClpB. **A**, **B**. Steady-state ATP hydrolysis was measured in reactions containing DnaK wild-type or mutant, DnaJ, GrpE, ClpB and heat-denatured malate dehydrogenase (MDH) and ATP as indicated and described in Methods. The fold stimulation above additive is calculated by dividing the rate of ATP hydrolysis for DnaK, J, E, B and MDH by the sum of the rates for DnaK, J, E, and MDH plus the rate for ClpB and MDH. DnaK subdomain IIB mutants are shown in **A** and subdomain IB and IIB loop mutants are shown in **B**. For **A** and **B** data are means \pm SEM (n=3).



Fig. 4.

Interaction between DnaK and ClpB in vivo and in vitro. **A.** A bacterial two-hybrid system was used to detect interactions between DnaK mutants and ClpB in vivo, as described in Methods. Fusion proteins were constructed between DnaK wild-type or mutant and one domain of *B. pertussis* adenylate cyclase, T25. ClpB was fused to the other domain, T18. The fusion proteins were coexpressed in BTH101 cells and the interaction between ClpB and DnaK was monitored by the expression of a reporter gene, β -galactosidase in liquid assays. Data are means \pm SEM (n=9–12) **B**–**C.** Interaction of DnaK (0.075 µM) with ClpB_{E279A,E678A} (ClpB_{trap}) (5 µM) in the presence of ATP was measured by fluorescence anisotropy using pyrene-maleimide labeled DnaK_{K106C} (DnaK_{py(Wt)}) or DnaK_{py} with additional substitutions (DnaK_{py(L257A)}, DnaK_{py(N282A*)}, DnaK_{py(R56A)} and DnaK_{py(Q57A*)}) as described in Methods. Data in **B** are presented as the fold change in signal upon addition of ClpB_{trap} to the reaction. In **C**, either GrpE or DnaK_{wt} (5 µM) was added to the DnaK_{py(wt)} (0.075 µM) and ClpB_{trap} (5 µM) reaction as indicated and the change in fluorescence anisotropy was measured. Data in **C** are presented as the change in

anisotropy signal (arbitrary units) upon addition of $ClpB_{trap}$ to the reaction. Data in **B**–C are means ± SEM (n=3).



Fig. 5.

The effect of GrpE on protein activation and remodeling by DnaK, DnaJ and ClpB. **A**, **C**. RepA was incubated with ATP, DnaJ and increasing concentrations of DnaK_{wt} in the presence of 0 μ M, 0.03 μ M or 2 μ M GrpE without ClpB (**A**) or with 180 nM ClpB (**C**), as indicated. RepA activation was measured as described in Methods. In **A** and **C**, gray dashed lines at 0.2 and 0.4 μ M DnaK are present to aid the eye. **B**, **D**. RepA was incubated with 0.4 μ M DnaK_{wt} (**B**) or 0.1 μ M DnaK_{wt} (**D**), DnaJ and ATP in the presence of increasing GrpE concentrations without (**B**) or with 20 nM ClpB (**D**) as indicated. RepA activation was measured as described in Methods. **E.** DnaK_{wt} (1.4 μ M) and DnaJ (0.25 μ M) were incubated with hdGFP and ATP in the absence or presence of ClpB (0.25 μ M) and GrpE as indicated. The increase in fluorescence intensity was monitored over time. Initial rates of reactivation of hdGFP were determined as described in Methods. Data in **A**–**E** are means ± SEM (n=3). Some error bars are hidden by symbols.



Fig. 6.

Substitutions in subdomains IB and IIB of the yeast Hsp70, Ssa1, cause defects in collaboration with Hsp104. A–C. Reactivation of heat-denatured luciferase (hdLuc) was measured over time as described in Methods. hdLuc was incubated with Ssa1_{wt} (2 μ M) (A–C) or Ssa1 mutant (2 μ M in A and C and 6 μ M in B), yeast Hsp40, Ydj1, (0.5 μ M when 2 μ M Ssa1 was used and 1.5 μ M when 6 μ M Ssa1 was used) and ATP in the presence or absence of 0.4 μ M Hsp104, as indicated. Data in A–C are means ± SEM (n=3). Some error bars are hidden by symbols.



Fig. 7.

Computational model of DnaK and ClpB indicating that one DnaK interacts with two ClpB protomers. **A**, **B**. Model of full-length ClpB hexamer shown as a top view (**A**) or a bottom view (**B**) bound to full-length DnaK (gray). The two ClpB protomers that interact with DnaK are shown in blue (chain E) and green (chain F). ClpB M-domain residues identified from modeling performed in this study as interacting with DnaK are shown in cyan (chain E) and yellow (chain F). ClpB NBD-1 residues identified as interacting with DnaK are shown in purple (chain E). DnaK residues identified as interacting with the ClpB chain E M-domain are shown in red and with ClpB chain F M-domain are in magenta (Supplemental Table S1). DnaK residues interacting with ClpB chain E NBD-1 are shown in orange. The black boxes indicate the region of interest that is shown larger in panels **C** and **D**. **C**, **D**. The region of interaction between ClpB M-domains and the DnaK NBD highlighted by the black boxes in **A** and **B** are enlarged, with colors as indicated in **A** and **B**. Models were obtained as described in Methods and images in **A–D** were made using PYMOL (www.pymol.org) ⁵⁷.

Table 1

Substitutions in the nucleotide-binding domain of DnaK

Name of Mutant ^a	Region of NBD	Substitution
K106A*	No known interactions	K106A, G107A, Q108A, K109A
K121E*		K121E, K122E, K124E, K125E
R159A*		R159A, I160A, E164A
E310K*		E310K, D311K, N314K
K351A*		K351A, K352A, A354G, E355A
Y145A*	DnaJ binding region	Y145A, N147A, D148A
K166A*		K166A, R167A, I169A
N187A*		N187A, T189A
E217A*		E217A, V218A
L49A	GrpE binding region	L49A
R56A		R56A
Q57A*		Q57A, V59A, T60A
L257A		L257A
R261C		R261C
N282A*		N282A, P284A, Y285A
A288C		A288C
A290C		A290C

 a_* indicates multiple amino acid substitutions

Table 2

Substitutions in the nucleotide-binding domain of Ssa1 or hHsp70

Substitution	Hsp70	Homologous residue in E. coli DnaK
	Ssa1	
L48A		L49
Q56A		Q57
R255A		L257
R259A		R261
D282A		P284
S283A		Y285
G287A		D289
D289A		H295
	Human Hsp70	
R262A		R261

Table 3

Residues involved in DnaK-ClpB interaction

Residue in DnaK	SASA ^a	DnaK	DnaK-ClpB hexamer complex	DnaK-GrpE complex
R56	Absolute SASA $(Å)^2$	199.0	21.7	10.7
	Relative ^b SASA	0.8	0.1	0
V59	Absolute SASA $(Å)^2$	84.2	58.1	13.8
	Relative SASA	0.5	0.4	0.1
T60	Absolute SASA $(Å)^2$	108.3	87.4	25.6
	Relative SASA	0.7	0.6	0.2
L257	Absolute SASA $(Å)^2$	89.1	65.7	8.6
	Relative SASA	0.5	0.4	0
R261	Absolute SASA $(Å)^2$	66.2	3.4	0
	Relative SASA	0.3	0.0	0
N282	Absolute SASA $(Å)^2$	9.06	95.2	35.4
	Relative SASA	0.6	0.6	0.2
P284	Absolute SASA $(Å)^2$	80.2	50.7	0.3
	Relative SASA	0.6	0.35	0
Y285	Absolute SASA $(Å)^2$	160.9	37.7	70.1
	Relative SASA	0.7	0.2	0.3
$a^{\text{SASA}} \equiv \text{solvent acc}$	essible surface area (see]	Methods)		

 $b_{\rm Relative}$ SASA values of ~1 suggest the residue is located on the surface of the protein