Mutagenesis Reveals the Complex Relationships between ATPase Rate and the Chaperone Activities of Escherichia coli Heat Shock Protein 70 (Hsp70/DnaK)*

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The Escherichia coli 70-kDa heat shock protein, DnaK, is a molecular chaperone that engages in a variety of cellular activities, including the folding of proteins. During this process, DnaK binds its substrates in coordination with a catalytic ATPase cycle. Both the ATPase and protein folding activities of DnaK are stimulated by its co-chaperones, DnaJ and GrpE. However, it is not yet clear how changes in the stimulated ATPase rate of DnaK impact the folding process. In this study, we performed mutagenesis throughout the nucleotide-binding domain of DnaK to generate a collection of mutants in which the stimulated ATPase rates varied from 0.7 to 13.6 pmol/µg/min⁻¹. We found that this range was largely established by differences in the ability of the mutants to be stimulated by one or both of the co-chaperones. Next, we explored how changes in ATPase rate might impact refolding of denatured luciferase in vitro and found that the two activities were poorly correlated. Unexpectedly, we found several mutants that refold luciferase normally in the absence of significant ATP turnover, presumably by increasing the flexibility of DnaK. Finally, we tested whether DnaK mutants could complement growth of ΔdnaK E. coli cells under heat shock and found that the ability to refold luciferase was more predictive of in vivo activity than ATPase rate. This study provides insights into how flexibility and co-chaperone interactions affect DnaK-mediated ATP turnover and protein folding.

One of the main roles of DnaK is to enable the folding of nascent or otherwise unfolded proteins (6). In this role, DnaK is thought to limit aggregation and facilitate folding by binding to the hydrophobic regions exposed in these substrates. Briefly, DnaK, like all the Hsp70 family members, consists of a substrate-binding domain (SBD) and a nucleotide-binding domain (NBD) connected by a hydrophobic linker (7–9). The NBD of DnaK is further divided into four subdomains as follows: IA/IIA, which form the base, and IB/IIB, which form the upper walls of the nucleotide binding cleft (Fig. 1A). The binding of ATP to DnaK results in an “open” conformation with low substrate affinity. Upon hydrolysis, the ADP-bound form assumes a “closed” conformation that binds substrate with higher affinity (10–16). Thus, allosteric communication between the two domains is thought to link nucleotide turnover to substrate binding and release.

DnaK alone has a low intrinsic ATPase rate, which facilitates regulation by the important co-chaperones, DnaJ and GrpE. DnaJ specifically stimulates ATP hydrolysis and thus favors high affinity substrate binding (17, 18). In addition, DnaJ independently binds to substrates with its C-terminal domains and is thought to help these proteins bind to DnaK (19, 20). GrpE, on the other hand, induces nucleotide exchange and leads to substrate release (21). These co-chaperone activities appear to be required for the cellular functions of DnaK because deletion of either DnaJ or GrpE causes defects in growth at elevated temperatures, similar to what is seen in ΔdnaK strains (5, 22, 23).

Many of the key insights into the folding of substrates by DnaK have emerged from in vitro studies on the model substrate, firefly luciferase. For example, it was found that DnaK requires DnaJ and GrpE to refold denatured luciferase (6). This platform has also been used to explore the roles of ATP hydrolysis in controlling substrate folding. For example, this process was found to require multiple cycles of ATP hydrolysis (5, 6). Also, ATPγS blocks refolding, further suggesting an important role for nucleotide cycling (24–26). However, truncated forms of DnaJ, which are able to stimulate ATP hydrolysis normally but cannot interact with substrates, are unable to stimulate luciferase refolding (18). Together, these results suggest that ATPase activity is necessary but not sufficient to achieve luciferase folding.

Finally, the chaperone function of DnaK can also be assayed in vivo by monitoring the ability of DnaK mutants to complement growth of ΔdnaK E. coli cells under heat shock (also called heat shock rescue) (27). At elevated temperatures, many pro-
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teins in E. coli become prone to unfolding and aggregation. One of the roles of DnaK is to bind these substrates, protecting them from aggregation (3, 28, 29). Following a return to normal temperature, DnaK also participates in active refolding (30, 31). Similar to what was observed in the in vitro luciferase refolding experiments, the ATPase activity of DnaK appears to be required during heat shock, because active site mutations that abolish nucleotide turnover are unable to rescue heat shock (24–26).

Taken together, these data suggest that ATPase rate may be an important modulator of chaperone activities. However, previous studies largely relied on either single mutations of catalytic residues or nucleotide mimics (24–26). Thus, it remains unknown if changes in the rate of ATP hydrolysis lead to predictable changes in chaperone functions, such as luciferase folding and heat shock rescue. Insights into these relationships have been complicated by several factors. First, the DnaK chaperone system is impacted by a number of potential variables. For example, several studies have highlighted substrate binding kinetics, interdomain communication, and the stimulation of the DnaK ATPase rate as important variables for refolding or cellular growth under heat shock (19, 32–40). Second, the interdependence of these variables presents important challenges. For example, a mutation that disrupts interdomain communication might also interfere with substrate binding and DnaJ-mediated ATPase stimulation (34, 41).

To better understand how the ATPase rate might regulate chaperone activity, we have undertaken a strategy of performing mutagenesis on the NBD of DnaK to generate a battery of mutants that varied in their co-chaperone-stimulated ATPase rates by over 15-fold. We envisioned that this collection of mutants might reveal the trends between turnover rates and folding outcomes. Moreover, one of our main goals in this study was to support our ongoing efforts to discover chemical compounds that target Hsp70s (24, 42, 43). As part of those studies, we wanted to evaluate if genetic modulation of the DnaK ATPase rate can predicitively modulate chaperone activities in vitro and in vivo. We anticipated that knowledge about these relationships would enable more predictive strategies for finding potent chemical probes for use in cellular and animal models.

Here, we report that ATP hydrolysis rate and luciferase refolding activity were poorly correlated. The disconnection between these two activities was most striking in a series of mutations that displayed negligible ATPase activity but retained normal refolding activity. Moreover, we found that, across the whole series of mutants, the in vitro refolding activity was more predictive of heat shock rescue than ATPase activity. These results provide insight into the function of the DnaK chaperone network.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following sources: Platinum Pfx DNA polymerase (Invitrogen); pMCSG7 plasmid (Midwest Center for Structural Genomics, Bethesda); ATP-agarose column (Sigma); NRLLLTG peptide (University of Michigan Peptide Core) (15); luciferase and SteadyGlo Reagent (Promega, Madison, WI); and ΔdnaK E. coli cells (a generous gift from Dr. Ursula Jacob). Furthermore, all absorbance and luminescence measurements were performed using a SpectraMax M5 ( Molecular Devices, Sunnyvale, CA).

Plasmids and Protein Purification—The E. coli dnaK and grpE genes were amplified by PCR using Platinum Pfx DNA polymerase and inserted into the pMCSG7 plasmid through ligation-independent cloning, as described previously (44). The partial overlapping site-directed mutagenesis primers for dnaK were designed based on the report of Zheng et al. (45), and mutagenesis of the dnaK gene was carried out following the user manual for the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Twenty nine dnaK mutants were made as follows: T12A, K55A, R56A, F67L, R71A, D85A, M89A, P90A, F91A, K106A, E171S, L177A, V192A, Y193A, G198A, T199A, I202A, S203A, E217A, G223A, L227A, G228A, E230A, E230Q, D231N, D233A, S234A, K263A, and I373A. The wild type (WT) His-tagged DnaK and its mutants were expressed in BL21(DE3) cells and first purified by batch purification with nickel-nitritriacetic acid His-Bind® resin (Novagen, Darmstadt, Germany) following the user manual. The His tag of eluted DnaK was then removed by His-tagged tobacco etch virus protease (1 mM DTT, 4 °C, overnight incubation). After adjusting the MgCl2 and KCl concentration to 10 mM, the sample was further purified by ATP-agarose column using previously established protocols (43). Finally, the remaining cleaved His tag was removed by nickel-nitritriacetic acid column. The purification of GrpE followed the same strategy except that the ATP-agarose column was excluded. DnaJ was purified as described previously (43) with the exclusion of hydroxypatite and Q-Sepharose fast-flow column purification steps and the addition of a Superdex 200 gel filtration column (GE Healthcare) to remove contaminating ATPase activity. Finally, N-terminal His-tagged J-domain 2–108 was purified by nickel-nitritriacetic acid column as described above without cleaving the His tag. All proteins were concentrated and exchanged into 25 mM Tris buffer (10 mM KCl (150 mM KCl for DnaJ), 5 mM MgCl2, pH 7.5) and stored at −80 °C until use.

Circular Dichroism—WT DnaK and mutants were prepared in 10 mM sodium phosphate buffer (100 mM sodium fluoride, pH 7.4) and spectra collected at 0.1 mg/ml in a 0.1-cm cuvette at room temperature. CD spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Easton, MD) at 1-nm intervals from 190 to 260 nm at a scanning speed of 50 nm/min and a 0.5 nm bandwidth. Each spectrum reported is the average of 15 scans after the subtraction of the base-line spectrum (buffer without the addition of DnaK) and normalization (millidegree cm2 dmol−1).

ATPase Activity—This procedure was adapted from a previously described protocol (43). Briefly, samples were prepared with the addition of DnaK, DnaJ, GrpE, NR substrate (NRLLLTG), and/or denatured luciferase to a total volume of 15 µl in each well. Next, 10 µl of 2.5 mM ATP was added to start the reaction. The final concentrations were as follows: ATP (1 mM), DnaK (0.5 µM), and NR substrate (100 µM), unless otherwise noted. Intrinsic ATPase rate was measured with DnaK (0.6 µM) in the absence of co-chaperones or substrate. When ATPase rate was tested as a comparison to refolding activity (see Figs. 2–4), guanidine hydrochloride-denatured luciferase
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at 8 nm and DnaK at 1 μM were used to match the conditions described in the luciferase refolding assay (see below). The final concentrations of DnaJ, GrpE, are reported for each experiment under ‘‘Results.’’ For steady state conditions, samples were incubated at 37 °C for 1–3 h, and then 80 μl of malachite green reagent was added to each well, immediately followed by 10 μl of 32% (w/v) sodium citrate. Samples were mixed thoroughly and incubated at 37 °C for 15 min. Finally, A620 was measured. All experiments were performed in triplicate, and the signal from nonspecific ATP hydrolysis in controls lacking DnaK was subtracted. A phosphate standard curve (using potassium dibasic phosphate) was generated each day and used to convert the units to pmol of P/μg of DnaK/min.

Stimulation curves were evaluated by fitting the data using a hyperbolic fit with a non-zero intercept as shown in Equation 1.

\[ y = \frac{V_{\text{max}}x}{(K_m + x)} + b \]  

(Eq. 1)

The nonlinear fit was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego).

Luciferase Refolding—The luciferase refolding activity of DnaK WT and mutants was evaluated as described with minor changes (24). Briefly, denatured firefly luciferase was prepared by beginning with a concentrated stock (8.2 μM) of luciferase with 6 M guanidine hydrochloride in 25 mM HEPES buffer (50 mM potassium acetate, 5 mM DTT, pH 7.2). This stock was incubated at room temperature for 1 h and then diluted to 0.2 μM with the same HEPES buffer without guanidine hydrochloride. This preparation was used as the stock solution for final sample preparation. Enzyme mix (10 μl) containing DnaK, DnaJ, GrpE, and denatured firefly luciferase in 39 mM HEPES buffer (20 mM potassium acetate, 1.7 mM magnesium acetate, 3 mM DTT, 0.1 M DTT, 12 mM creatine phosphate, 50 units/ml creatine kinase, pH 7.6) was first added into each well, and then 4 μl of 3.5 mM ATP, dissolved in water, was added to start the reaction. The final concentration of DnaK was 1 μM, denatured luciferase was 8 nM, and ATP was 1 mM unless otherwise noted. The concentration of DnaJ and GrpE is reported for each experiment under “Results.” After 1 h of incubation at 37 °C, equilibrium was reached, and 14 μl of ATPase Rate Is Not Predictive of DnaK Chaperone Function

Complementation of the Heat Shock Phenotype in a ΔdnaK Strain—Thermosensitive E. coli ΔdnaK (DE3) cells that expressed target genes under the T7 promoter were generated using ADE3 lysogenization kit (Novagen) based on the reported method of Sugimoto et al. (46). pMCSG7 plasmids containing dnaK WT and mutant genes were transformed into ΔdnaK (DE3) E. coli cells, and transformation with an empty pMCSG7 vector served as a negative control. For the complementation assay, a single colony of each dnaK mutant was inoculated into 5 ml of LB with 50 μg/ml ampicillin and grown with shaking overnight at 30 °C. The next day, all the overnight cultures were diluted to A600 = 0.055 by LB containing 50 μg/ml ampicillin and 4 μM isopropyl 1-thio-β-D-galactopyranoside. From the diluted culture, a 100-μl aliquot was loaded onto a transparent 96-well flat bottom plate (Corning Glass) in triplicate. These samples were incubated at 43 °C with shaking for 6 h, and the A600 value for each well was measured. The expression of DnaK protein in each clone was verified by adding 4 μM isopropyl 1-thio-β-D-galactopyranoside to the undiluted overnight culture, incubating at 37 °C for 5 h, lysing the cultures, normalizing the protein content, and then separating proteins by SDS-PAGE.

RESULTS

Design of DnaK Mutants—In this study we aimed to assess whether changes in the ATPase rate of DnaK lead to predictable changes in chaperone function. Toward this goal, we employed available mutational and structural data to select ~30 novel or established mutations in the NBD of DnaK (8, 41, 47–49). We avoided mutating the SBD to minimize direct disruption of substrate binding. Rather, these mutations were specifically designed to impact ATP turnover by four potential mechanisms (Fig. 1B). Known DnaK mutations, E171S and T199A, which alter residues in the ATP-binding pocket, made up class I. Class II included L177A and I373A, which were predicted to disrupt DnaJ-mediated ATPase stimulation based on homology to mutations made in other Hsp70 family members (14, 34, 41). Two mutants, V192A and Y193A, which are located near the proposed DnaJ-binding site, were also included in class II. Class III included K55A and R56A, which are known to disrupt binding to GrpE (8, 50), and 10 additional mutants in the IB and IIB subdomains that we hypothesized might impact nucleotide exchange. Finally, class IV mutations included residues around the proposed hinge region (residue 225–230) at the IIA/IIB subdomain interface. This region is proposed to undergo an ATP-induced structural change (47, 48). Together, this collec-
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As mentioned above, the ATPase rate of DnaK is stimulated by three main factors as follows: DnaJ, GrpE, and substrate. Thus, we wanted to measure the ability of these factors to stimulate each of the DnaK mutants. Toward this goal, we calculated the $K_m$ and $V_{max}$ values for DnaJ, GrpE, and a model DnaK substrate, NRLLLTG (Table 1 and supplemental Fig. 3). From these experiments, we found that the mutants varied in their responses to these stimuli. Using $K_m$ to estimate binding to DnaK, we found that most mutants varied in their affinity for DnaJ by $\sim 10$-fold (0.50 to 5.4 $\mu$m). In addition, a few mutants, such as E217A and L227A, had very weak stimulation, which precluded fitting of the curves (Table 1 and supplemental Fig. 3). Similarly, the $K_m$ value of GrpE stimulation varied over a range of almost 100-fold, with multiple mutants unable to be stimulated. Finally, maximum stimulation ($V_{max}$) varied by $\sim 6$-fold for DnaJ, 7-fold for GrpE, and 6-fold for substrate. Thus, we found that this collection had the desired wide range of $K_m$ and $V_{max}$ values for co-chaperone and substrate-mediated ATPase stimulation. Based on these findings, we reasoned that they could be used to probe the correlation between ATPase rate and luciferase folding.

ATPase Rate and Refolding Activity of DnaK Mutants Are Weakly Correlated—One of the functions of DnaK is to refold damaged proteins, and this activity can be measured in vitro by luciferase refolding assays. In this experiment, chemically denatured firefly luciferase is diluted in the presence of DnaK, ATP, DnaJ, and GrpE. This chaperone system gradually restores the misfolded luciferase, and the refolding process can be monitored by an increase in luminescence. To directly explore the correlation between stimulated ATPase activity and chaperone-mediated refolding, we recorded both activities for each DnaK mutant at the same concentrations of DnaK, DnaJ, GrpE, luciferase, and ATP (supplemental Fig. 4).

To explore potential correlations in these two activities of DnaK, we plotted the refolding activity against ATPase rate for each mutant (Fig. 2A). This analysis revealed poor correlation ($R^2 = 0.22$), between the activities. A closer inspection of these results revealed some additional trends. For example, many mutants, such as T12A, had a higher ATPase rate than WT, but this enhanced turnover did not lead to correspondingly higher refolding activity. Furthermore, some mutants, such as R56A, had the same ATPase rate as WT but decreased refolding activity. Curiously, there were also mutants such as F67L, P90A, F91A, E230Q, D231N, and K263A, which had significantly lower ATP hydrolysis rates than WT DnaK, but they retained normal luciferase refolding activity. We named these DnaK mutants “decoupling mutants.”

Because we found that many of the DnaK mutants had altered rates of intrinsic ATPase activity, we next analyzed the correlations between these values and luciferase refolding. Consistent with the previous findings, the intrinsic ATPase rates had almost no correlation with luciferase refolding activities ($R^2 = 0.007$; supplemental Fig. 5). Similarly, the ATPase rates of the mutants stimulated by DnaJ alone (i.e. no GrpE or substrate) also failed to correlate ($R^2 = 0.071$; supplemental Fig. 5).

We were particularly interested in exploring the activities of the decoupling mutants in greater detail. Interestingly, when the mutants were classified based on their locations, the decoupling mutants clustered on the IB and IIB subdomains. Most mutations in the IB and IIB subdomains had normal refolding activity regardless of ATPase rate ($R^2 =$...
Correspondingly, when only the IA/IIA subdomain mutants were examined, the overall ATPase rates became more positively associated with refolding activities ($R^2 = 0.55$) (Fig. 2B).

Decoupling Mutants of the IB Subdomain Make DnaK More Flexible—Further analysis revealed that the decoupling mutants in the IB subdomain, F67L, P90A, and F91A, were co-localized in a hydrophobic patch adjacent to loop(74–96) (Fig. 3A). Loop(74–96) has been shown to be crucial for cooperation with DnaJ and GrpE during both refolding and heat shock rescue (49). Consistent with this important role, loop(74–96) is connected to a small helix containing Lys-70, a residue that is essential for ATP hydrolysis (51). Based on this analysis, we hypothesized that mutations to the hydrophobic triad of Phe-67, Pro-90, and Phe-91 might destabilize loop(74–96). In turn, this increased flexibility might disrupt positioning of Lys-70, which would explain the low ATPase rates. In support of this hypothesis, we found that the three decoupling mutants were more sensitive to trypsin digestion and, furthermore, that they exhibited a different digestion pattern than WT or other IB subdomain mutations (Fig. 3B and supplemental Fig. 6). This result suggests that, through increased flexibility, the IB subdomain decoupling mutants may allow for productive interactions with misfolded lucif-

### TABLE 1

ATPase rates of the DnaK mutants

| DnaK   | Intrinsic ATPase rate | DnaJ Stimulation$^b$ | GrpE Stimulation$^c$ | Substrate Stimulation |
|--------|-----------------------|----------------------|----------------------|----------------------|
|        | Vmax$_p$/pmolP/μg/min | Km$_p$/μM            | Vmax$_E$/pmolP/μg/min | Km$_E$/μM            | Vmax$_μ$/pmolP/μg/min | Km$_μ$/μM |
| WT     | 1.7 ± 0.2             | 0.58 ± 0.12          | 47.2 ± 4.0           | 108.3 ± 29.0         | 6.0 ± 0.9             | 90 ± 28   |
| E171S  | 1.2 ± 0.1             | NF$^a$               | NF                   | NF                   | NF                   | NF        |
| T199A  | 0.6 ± 0.2             | NF                   | NF                   | NF                   | NF                   | NF        |
| L177A  | 3.1 ± 0.3             | 8.8 ± 1.1             | 1.25 ± 0.50          | 19.5 ± 1.7           | 47.8 ± 16.2           | 5.4 ± 0.2  |
| V192A  | 3.8 ± 1.0             | 6.7 ± 0.4             | 0.53 ± 0.13          | 84.2 ± 5.6           | 114.3 ± 23.5          | 5.0 ± 0.1  |
| Y193A  | 5.0 ± 0.3             | 13.4 ± 0.5            | 0.81 ± 0.12          | 57.0 ± 3.2           | 94.3 ± 17.4           | 7.0 ± 0.6  |
| I373A  | 3.7 ± 1.2             | 2.3 ± 0.7             | 26.4 ± 4.7           | 188.8 ± 82.9         | NF                   | NF        |
| T12A   | 2.9 ± 0.4             | 40.5 ± 2.7            | 2.57 ± 0.39          | 11.7 ± 1.9           | 53.7 ± 34.6           | 7.1 ± 0.1  |
| K55A   | 2.5 ± 0.4             | 30.4 ± 2.5            | 3.25 ± 0.54          | NF                   | NF                   | NF        |
| R56A   | 3.2 ± 0.3             | 19.5 ± 1.2            | 3.53 ± 0.42          | 64.2 ± 15.5          | 544.4 ± 210.2         | 13.3 ± 0.4 |
| F67L   | 1.4 ± 0.3             | NF                   | NF                   | NF                   | NF                   | NF        |
| R71A   | 2.4 ± 0.3             | NF                   | NF                   | NF                   | NF                   | NF        |
| D85A   | 3.0 ± 0.3             | 14.1 ± 1.1            | 0.82 ± 0.22          | 17.4 ± 2.6           | 35.8 ± 21.5           | NF        |
| M89A   | 3.5 ± 0.2             | 12.7 ± 0.9            | 0.56 ± 0.14          | 44.7 ± 17.4          | 364.6 ± 263.8         | 8.2 ± 0.2  |
| P90A   | 1.8 ± 0.4             | 13.8 ± 4.4            | 4.3 ± 2.5            | 15.4 ± 1.9           | 5.2 ± 2.2             | NF        |
| F91A   | 1.6 ± 0.4             | NF                   | NF                   | NF                   | NF                   | NF        |
| K106A  | 5.0 ± 0.4             | 14.9 ± 0.6            | 0.96 ± 0.14          | 46.4 ± 4.6           | 64.5 ± 23.6           | 6.3 ± 0.1  |
| D233A  | 5.7 ± 0.4             | 41.6 ± 1.4            | 1.15 ± 0.13          | NF                   | NF                   | NF        |
| K253A  | 3.4 ± 1.0             | NF                   | NF                   | NF                   | NF                   | NF        |
| G198A  | 2.2 ± 0.1             | 10.4 ± 0.6            | 0.50 ± 0.11          | NF                   | NF                   | NF        |
| I202A  | 1.5 ± 0.1             | 13.2 ± 0.8            | 0.67 ± 0.14          | NF                   | NF                   | NF        |
| S203A  | 3.9 ± 0.1             | NF                   | NF                   | 29.7 ± 0.7           | 6.9 ± 0.6             | 2.1 ± 0.2  |
| E217A  | 1.2 ± 0.1             | NF                   | NF                   | NF                   | NF                   | NF        |
| G223A  | 0.5 ± 0.1             | NF                   | NF                   | NF                   | NF                   | NF        |
| L227A  | 1.0 ± 0.1             | NF                   | NF                   | NF                   | NF                   | NF        |
| G228A  | 2.5 ± 0.1             | NF                   | NF                   | NF                   | NF                   | NF        |
| E230Q  | 0.6 ± 0.4             | 14.1 ± 1.0            | 1.1 ± 0.73           | NF                   | NF                   | NF        |
| E230A  | 3.2 ± 0.4             | 14.3 ± 1.8            | 1.95 ± 0.65          | 12.5 ± 1.5           | 37.8 ± 18.3           | NF        |
| D231N  | 0.6 ± 0.1             | NF                   | NF                   | NF                   | NF                   | NF        |
| S234A  | 0.5 ± 0.1             | 9.9 ± 0.7             | 0.32 ± 0.09          | NF                   | NF                   | NF        |

$^a$ The gray boxes (NF) indicate that either ATPase rate was not stimulated, as defined by a mutant in which the S.E. of $K_m$ or $V_{max}$ encompassed zero, or when a nonlinear fit could not be obtained.

$^b$ DnaJ stimulation was tested in the presence of NR substrate (100 μM).

$^c$ GrpE stimulation was tested in the presence of both DnaJ (1 μM) and NR substrate (100 μM).

$^d$ The $V_{max}$ value (calculated by Equation 1 under the “Experimental Procedures”) represents the increase in ATPase rate compared with solvent control. Raw data can be found in supplemental Fig. 2. Error is mean ± S.E.
erased without relying on structural transitions normally linked to ATP hydrolysis.

Because loop(74–96) was reported to be involved in interactions with DnaJ (49), we wanted to specifically test the ability of the decoupling mutants to be stimulated by this co-chaperone in the presence of denatured luciferase. In ATPase assays, we varied the concentration of DnaJ and found that F67L, P90A, and F91A resulted in higher ATPase activities than WT DnaK (Fig. 3, A, B, and C). The final concentrations were as follows: DnaK (1 mM), DnaJ (0.25 μM), GrpE (0.125 μM), denatured luciferase (8 nM), and ATP (1 mM). B and C, analysis of subsets of mutations based on their locations. Mutations in the IA/IIA subdomains (8) have a higher correlation (R² = 0.55) than those in the IB/IIB subdomains (C, R² = 0.04). B and C, IA/IB subdomain mutants are shown as gray squares, IIA/IIB subdomain mutants as black squares, and WT as black circles. Although WT activity is shown in B and C, it was not included for the calculation of R² value. Each data point is the average of triplicates, and the error bars represent the mean ± S.E. A.U., arbitrary units.

Asp-231, were located adjacent to each other in the IIB subdomain (Fig. 4A). This region is at the interface of the IIB and IIA subdomains, where a hinge region (residue 225 to 230 in DnaK) is thought to rotate the IIB subdomain away from the IB subdomain upon ATP hydrolysis (47). This conformational change has been observed in GrpE-bound DnaK, and it was previously proposed to be important for nucleotide release (8). To explore the basis for the observed decoupling by mutations E230Q and D231N, we measured the stimulation of these mutants by DnaJ and GrpE. In ATPase assays, the decoupling mutants E230Q and D231N had similar ATPase rates compared to WT (Fig. 3, A, B, and C). However, unlike WT DnaK, the ATPase activities of these mutants were not stimulated by GrpE and, rather, were significantly inhibited by a low concentration of this co-chaperone (~0.1 μM) (Fig. 4C). When these same mutations were tested in the refolding assay, they responded to GrpE in a manner similar to WT (Fig. 4D). Thus, in these mutants, ATPase rate was decoupled from refolding largely by affecting the GrpE-mediated stimulation of these activities.

**Refolding Activity of DnaK Mutants Was More Predictive of in Vivo Function**—Because this collection of DnaK mutants had revealed that ATPase rate and luciferase refolding activity were only weakly correlated, we wanted to explore which activity, if any, would be most predictive of a cellular function of DnaK. Toward this goal, we explored the ability of the DnaK mutants to protect against heat shock in ΔdnaK (DE3) E. coli strains lacking dnaK are temperature-sensitive and unable to grow after exposure to elevated temperature. As expected, we found that WT DnaK, expressed from an inducible plasmid, could complement growth of ΔdnaK E. coli at 43 °C (supplemental Fig. 7A). Using the same procedure, we then tested each of the DnaK mutants. First, plasmids for each mutant were transformed into ΔdnaK (DE3) E. coli cells, and protein expression was confirmed (supplemental Fig. 7B). Following heat shock at 43 °C, the A₅₀₀ was measured, and these results were plotted against the ATPase or refolding activity of each mutant (Fig. 5, A and B). From this analysis, we found a strikingly poor correlation between ATPase rate and heat shock rescue (R² = 0.15). However, refolding activity was relatively better correlated (R² = 0.40).
Potential changes in DnaK activity and refolding efficiency have been observed with the expression of the NBD or C-terminal half of DnaJ (18–20, 59). Although the 1–104-residue fragment of DnaK can fully stimulate ATP hydrolysis, it cannot stimulate the DnaK refolding activity in vivo (50, 55, 56). Previous data have suggested that ATP hydrolysis is necessary but not sufficient for luciferase refolding (16, 22–24). To explore this finding more deeply, we examined if the ATPase rate of DnaK could predict its luciferase refolding activity using a series of mutants. In the 29 DnaK mutants tested, we found that the two activities were poorly correlated. This result suggests that additional factors might contribute to refolding.

To explore what some of these contributing factors might be, we specifically investigated how the mutants were stimulated by co-chaperones and substrate (see Table 1 and Fig. 2). This analysis showed that mutants unable to refold luciferase, such as E171S, T199A, and G228A, were also defective in their ability to be stimulated by DnaJ, GrpE, and substrate in the ATPase assay. However, the converse was not necessarily true. For example, there were several mutants whose ATPase rate could not be stimulated, yet they still had the same refolding activity as WT DnaK. These results demonstrated that the relationship between ATPase and luciferase refolding activity is complex. More specifically, these results suggested that the ability of DnaK to be stimulated by DnaJ, GrpE, or peptide substrate is not necessarily predictive of its activity in the refolding assay. This conclusion is illustrated by several mutants in which drastic changes in both the $V_{max}$ and $K_m$ values of DnaJ-mediated ATPase stimulation were observed without a significant impact on refolding activity (see Table 1 and Fig. 3). Most notably, the IB subdomain decoupling mutants, F67L, P90A, and F91A, exhibited WT-like dependence on DnaJ for refolding, but had increased $K_m$ values for DnaJ-mediated stimulation of ATP hydrolysis. This observation suggests that DnaJ might promote ATPase and refolding activities by different mechanisms. In support of this model, DnaJ mutants that stimulate the ATPase rate of DnaK, but not its refolding activity, have been reported (17, 18, 57–59). The structure of DnaJ might provide a clue into how these two activities can be separated. Residues 1–104 of DnaJ are essential for interactions with the NBD of DnaK. This region is composed of the J-domain and a glycine- and phenylalanine-rich region. The C-terminal half of DnaJ contains two zinc-binding sites and a domain important for binding to substrate (18–20, 59). Although the 1–104-residue fragment of DnaJ can fully stimulate ATP hydrolysis, it cannot stimulate the

**DISCUSSION**

Relationship between ATPase and Refolding Activity of DnaK—As an isolated domain, the SBD of DnaK binds to substrate with the same affinity as ADP-bound DnaK and can slowly refold luciferase (52). Thus, one major role of the NBD seems to be to power the transition of the SBD between open and closed states, which accelerates binding-and-release of substrates and increases refolding activity (14, 34, 53, 54). Based on these findings, refolding and ATP turnover are thought to be closely linked functions of DnaK. Consistent with this idea, the co-chaperones DnaJ and GrpE stimulate both the ATPase rate and refolding activity of DnaK (50, 55, 56). Previous data have suggested that ATP hydrolysis is necessary but not sufficient for luciferase refolding (16, 22–24). To explore this finding more deeply, we examined if the ATPase rate of DnaK could predict its luciferase refolding activity using a series of mutants. In the 29 DnaK mutants tested, we found that the two activities were poorly correlated. This result suggests that additional factors might contribute to refolding.

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refolding activity of DnaK (18). More strikingly, Karzai and McMacken (17) found that DnaJ lacking its second zinc-binding site had normal affinity for denatured luciferase and actively stimulated ATP hydrolysis by DnaK, but this mutant was unable to transfer substrate to DnaK or support luciferase refolding. Those previous reports and our studies suggest that a secondary event, perhaps substrate presentation, may be important for refolding in the DnaK-DnaJ pair. We speculate that the decoupling mutations might interrupt ATPase activity without affecting DnaJ-mediated substrate presentation. However, this model lacks structural support, and the detailed mechanisms await further analysis.

It is important to note that other factors may also help to account for the lack of correlation between ATPase rate and refolding activity. These factors might include substrate-binding kinetics, nucleotide-dependent conformational changes, and co-chaperone complex formation (60–63).

Mechanisms of the IB and IIB Decoupling Mutants—We were particularly interested in understanding the mechanisms of the “decoupling” mutants, because this is, to our knowledge, the first report of DnaK mutants that have greatly decreased ATPase rates but normal refolding activities. Three of the decoupling mutants were mapped to a hydrophobic patch on the IB subdomain that appears to stabilize loop(74–96) and therefore regulate the position of Lys-70, a key residue for ATP hydrolysis. These decoupling mutants had low ATPase rates, yet they retained both normal refolding activities and the ability to restore growth under heat shock in E. coli. We hypothesize that the unusual properties of these mutants might arise from their increased flexibility, as measured by trypsin susceptibility. Specifically, this flexibility might allow the mutants to sample the open and closed conformational states that facilitate refolding. To date, there is no structure of open ATP-bound full-length Hsp70, but based on the structure of Hsp110, a member of the Hsp70 superfamily, the α-helical lid of the SBD interacts with IA and IB subdomains (64, 65). Therefore, it is tempting to speculate that the same interactions stabilize the open structure in DnaK. Moreover, this interaction of the SBD with subdomains IA and IB might create a barrier for switching between open and closed conformations. In this model, F67L, P90A, and F91A mutants, by destabilizing or otherwise repositioning loop(74–96), might reduce the barrier to conformational flexibility in the SBD, essentially mimicking the activity normally reserved for nucleotide hydrolysis. The net effect of this change might be to partially decouple ATP turnover from refolding activities. Further structural investigations of WT DnaK and these mutants may provide additional insight.

Interestingly, loop(74–96) is not present in Gram-positive bacteria. Moreover, the DnaK from Tetragenococcus halophilus, a Gram-positive bacterium, has excellent holdase activity, but its refolding and ATPase activities are not stimulated by T. halophilus DnaJ or GrpE (66). Furthermore, deletion of this loop from E. coli DnaK renders it unable to cooperate with co-chaperones in both ATP hydrolysis and luciferase refolding (49). These results further suggest that loop(74–96) may play a role in the allosteric regulation of DnaK.

The mutations in the IIB subdomain, E230Q and D231N, also appear to decouple ATPase and refolding activities but by a mechanism that is distinct from that used by the IB subdomain decoupling mutations. The IIB residues are co-localized at the interface between the IIA and IIB subdomains. This interface is particularly important for the allosteric regulation of DnaK.

Therefore, to date, there is no structure of open ATP-bound full-length Hsp70, but based on the structure of Hsp110, a member of the Hsp70 superfamily, the α-helical lid of the SBD interacts with IA and IB subdomains (64, 65). Therefore, it is tempting to speculate that the same interactions stabilize the open structure in DnaK. Moreover, this interaction of the SBD with subdomains IA and IB might create a barrier for switching between open and closed conformations. In this model, F67L, P90A, and F91A mutants, by destabilizing or otherwise repositioning loop(74–96), might reduce the barrier to conformational flexibility in the SBD, essentially mimicking the activity normally reserved for nucleotide hydrolysis. The net effect of this change might be to partially decouple ATP turnover from refolding activities. Further structural investigations of WT DnaK and these mutants may provide additional insight.

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The mutations in the IIB subdomain, E230Q and D231N, also appear to decouple ATPase and refolding activities but by a mechanism that is distinct from that used by the IB subdomain decoupling mutations. The IIB residues are co-localized at the interface between the IIA and IIB subdomains. This interface is proposed to contain a hinge region that controls the rotation of the IIB subdomain away from the IB subdomain upon ATP hydrolysis (47, 48). Decoupling mutations, E230Q and D231N,
ATPase Rate Is Not Predictive of DnaK Chaperone Function

A

In vitro refolding activity is more predictive of in vivo heat shock rescue

B

In vitro ATPase rate is less predictive of in vivo heat shock rescue

which are located on an α-helix adjacent to this flexible hinge, exhibited normal refolding and DnaJ-stimulated ATPase activities. However, these mutants had an altered response to GrpE. Specifically, GrpE failed to stimulate the ATPase activity of these mutants; moreover, it inhibited turnover at concentrations greater than -0.1 μM. Because GrpE binding also induces rotation of the IIB subdomain away from the IB (8), it is possible that E230Q and D231N interfere with the proper movement or positioning of the α-helix in response to interaction with GrpE. However, this change does not appear to prevent the conformational changes required for refolding, because the GrpE-stimulated folding activity was unchanged.

Together, our findings with both the IB and IIB decoupling mutants suggest that alterations in co-chaperone-mediated substrate binding and folding) that could potentially be used to understand in vivo functions (e.g. heat shock rescue, anti-apoptotic activity in cancer cells, and modulation of protein aggregation) (4, 68). Yet, it is not readily obvious which in vitro activity of Hsp70 is most predictive of a given in vivo function or whether all in vivo functions are equally dependent on a given measurable activity. Future work in this area may improve our ability to understand the connection between in vitro and in vivo functions of Hsp70 and thereby lead to a deeper mechanistic understanding of the Hsp70 chaperone system.

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