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Subunit interactions influence the biochemical and biological properties of Hsp104

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Contributed by Susan L. Lindquist, November 30, 2000

Point mutations in either of the two nucleotide-binding domains (NBD) of Hsp104 (NBD1 and NBD2) eliminate its thermotolerance function in vivo. In vitro, NBD1 mutations virtually eliminate ATP hydrolysis with little effect on hexamerization; analogous NBD2 mutations reduce ATPase activity and severely impair hexamerization. We report that high protein concentrations overcome the assembly defects of NBD2 mutants and increase ATP hydrolysis severalfold, changing V max with little effect on K m. In a complementary fashion, the detergent 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate inhibits hexamerization of wild-type (WT) Hsp104, lowering V max with little effect on K m. ATP hydrolysis exhibits a Hill coefficient between 1.5 and 2, indicating that it is influenced by cooperative subunit interactions. To further analyze the effects of subunit interactions on Hsp104, we assessed the effects of mutant Hsp104 proteins on WT Hsp104 activities. An NBD1 mutant that hexamerizes but does not hydrolyze ATP reduces the ATPase activity of WT Hsp104 in vitro. In vivo, this mutant is not toxic but specifically inhibits the thermotolerance function of NBD1 mutant that hexamerizes but does not hydrolyze ATP reduces the ATPase activity of WT Hsp104 in vitro. In vivo, this mutant is not toxic but specifically inhibits the thermotolerance function of WT Hsp104. Thus, interactions between subunits influence the ATPase activity of Hsp104, play a vital role in its biological functions, and provide a mechanism for conditionally inactivating Hsp104 function in vivo.

The HSP100/Clp family of chaperone proteins plays a wide variety of important cellular roles in different organisms, including survival of environmental stress, regulation of genetic competence, transposition, proteolysis, and control of a protein-based genetic element (prion). These seemingly unrelated roles are unified by a common remarkable biochemical mechanism: the proteins promote the disassembly of aggregated proteins and higher-order protein complexes (reviewed in refs. 1 and 2). Several biochemical properties are shared by HSP100 proteins and are required for their biological functions, suggesting that these properties are important to the complex phenomenon of protein disassembly. These properties include an ATP-hydrolyzing activity (3–10) and the ability to self-assemble into oligomers, primarily hexamers (11–13).

A member of the HSP100 family from Saccharomyces cerevisiae, Hsp104, is critical for survival after exposure to extreme temperatures (50°C) (14) or high concentrations of ethanol (20%) (15). These stresses cause protein denaturation, and Hsp104 promotes survival by facilitating the resolubilization of heat-damaged, aggregated proteins (reviewed in refs. 1 and 2). At normal temperatures, Hsp104 plays a critical role in the inheritance of the novel proteinaceous genetic element [PSI+] (often called a yeast prion), an ordered aggregate of the [PSI+] element (16).

Hsp104 is a member of the class 1 HSP100 proteins, with two distinct but highly conserved nucleotide-binding domains (NBDs) (1, 17). NBD1 and NBD2 of Hsp104 show only 22% amino acid identity with each other, yet each shares 40–60% identity with the corresponding domains of their Escherichia coli relatives, ClpA and ClpB (18). Although NBD1 and NBD2 are very different from each other, both contain classic Walker-type consensus sequences for the P-loop (19) that resemble those of NSF and the P-type transporter protein families. ATP promotes the assembly of Hsp104 into hexamers (11), and Hsp104 hydrolyzes ATP with a high degree of specificity (10). The mechanism by which Hsp104 performs its functions is not understood. However, both its ATPase activity and its oligomerization properties seem to be essential. Point mutations that perturb these functions (10, 11) eliminate the ability of Hsp104 to provide thermotolerance (20) and interfere with the normal metabolism of the [PSI+] element (16).

Previously, we reported that canonical P-loop residue mutations in NBD1 of Hsp104 virtually eliminate ATP hydrolysis but have little effect on oligomerization. In contrast, analogous mutations in NBD2 inhibit but do not eliminate ATP hydrolysis and severely impair oligomerization (10, 11). Based upon the crystal structures and mutational analysis of other Walker-type ATP binding sites (21), these mutations were chosen to inhibit ATP binding without perturbing the general structure of the domain. The related E. coli protein, ClpA, is similar to Hsp104 without analogous point mutations in NBD1 and NBD2 differentially affect oligomerization and ATP hydrolysis (22, 23). Curiously, however, the effects of the mutations are reversed from those reported for Hsp104 (10). Either the two domains have independent functions (one responsible for ATP hydrolysis, the other for oligomerization) and these functions have switched in the two proteins during the course of evolution or both domains contribute to ATP hydrolysis and oligomerization in a complex, interdependent manner, and other differences between the two proteins idiosyncratically cause analogous mutations to perturb one of these characteristics more than the other. Indeed, mutations that abolish oligomerization in Hsp104 or in ClpA also reduce ATP hydrolysis (10, 22, 23), suggesting a relationship between oligomerization and ATP hydrolysis. However, no kinetic information is available relating these properties in any HSP100 protein nor relating subunit interactions to biological function.

Here, we demonstrate that interactions between Hsp104 subunits increase the ATPase activity of Hsp104 in a cooperative fashion. We also report that Hsp104 proteins that carry point mutations in NBD1 or NBD2 profoundly alter the activities of wild-type (WT) Hsp104 in mixed oligomers and do so in different ways both in vitro and in vivo. The data support previous suggestions of functional distinctions in the two NBDs, illustrate the importance of proper subunit interactions in establishing the functional state of Hsp104, and provide different ways to alter that state.

Materials and Methods

Plasmids. Point mutations in HSP104 were produced and placed into expression vectors as described (10). Mutant HSP104 coding

Abbreviations: NBD, nucleotide-binding domain; WT, wild-type; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate.

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sequences were inserted behind the yeast glyceraldehyde-3-phosphate dehydrogenase promoter in pRS305 for integration at the LEU2 locus. The WT HSP104 coding sequence was placed under Gal1 regulation in pRS316 (cen6/ars4, URA3; 104hU). pHCA/GAL4(1–93), ER.VP16, which encodes a β-estradiol-activated inducer of Gal1-regulated genes (24), was the gift of D. Picard. Late in the course of these studies, we found that one of the mutants, K218T, had acquired an additional mutation producing K218T:A315T. This mutant behaved similarly to K218T alone when retested for assembly and ATP hydrolysis.

**Strains.** The integration constructs described above and their parent vector, pRS305 (cleaved with AflII), were integrated into yeast strain MD104–1U in which 80% of the HSP104 coding sequence is deleted. These strains were subsequently transformed with two plasmids: pHCA/GAL4(1–93), ER.VP16 and either pRS316 or 104hU. This generated strains with the following combinations of integrated glyceraldehyde-3-phosphate dehydrogenase-regulated (constitutive)/plasmid β-estradiol-regulated genes: vector/vector, K218T:A315T/vector, vector/WT, K218T:A315T/WT, K620T/vector, and K620T/WT.

**Protein Purification.** Hsp104 was purified from yeast and E. coli as described (11, 25). To compare mutant and WT, proteins were first dialyzed against and concentrated in 20 mM Heps, pH 7.5/140 mM KCl/15 mM NaCl/10 mM MgCl2/2 mM DTT using Ultrafree-15 centrifugal filter devices with M1 cut-off at 30,000 Da (Millipore). After concentrations were determined (25), proteins were diluted in the same buffer.

**ATPase Assays.** To relate ATP hydrolysis to oligomerization, both types of assay were performed in reaction buffer 1 (20 mM Heps, pH 7.5/140 mM KCl/15 mM NaCl/10 mM MgCl2). All reactions included 5 mM ATP (pH 7.5) except for Km experiments, where the reaction buffer, containing 5 mM MgCl2, included Mg-ATP (0.01 to 40 mM) ATP and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were adjusted to pH 7.5.

All assays shown were performed, as described (10), for times at which the phosphate released was linear for the assay system. Released Pi was quantified with malachite green (26), and the phosphate released was linear for the assay system. The phosphate dehydrogenase promoter in pRS305 for integration at the pHCA/GAL4(1–93), ER.VP16, which encodes a β-estradiol-activated inducer of Gal1-regulated genes (24), was the gift of D. Picard. Late in the course of these studies, we found that one of the mutants, K218T, had acquired an additional mutation producing K218T:A315T. This mutant behaved similarly to K218T alone when retested for assembly and ATP hydrolysis.

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All assays shown were performed, as described (10), for times at which the phosphate released was linear for the assay system. Released Pi was quantified with malachite green (26), and Km was determined with a Molecular Devices Vlmax kinetic microplate reader with SOFTMAX software. Values were calibrated against KH2PO4 standards and corrected for phosphate released in the absence of Hsp104. For each point, at least three and typically six independent assays were used to calculate the mean and standard deviation. The curves shown and Km and Vlmax values (500 μM to 25 mM ATP) were generated by using least squares fitting to the Michaelis–Menten equation with the KALEIDAGRAPH graphics program (Synergy Software, Reading, PA). Similar values were obtained when Lineweaver–Burke and Eadie–Hofstee plots were used. For calculation of the Hill equation were generated by plotting the rate of hydrolysis μg−1 protein versus the concentration of ATP using the nonlinear allosteric kinetic equation of the GRAFIT 4 graphics program (Erithacus Software, Surrey, U.K.).

Similar results were obtained with a second assay for which 5 μCi of [α-32P]ATP (3,000 MBq) was added per 25-μl reaction; reactions were stopped by addition of formic acid to 1.5 M; ATP and ADP were resolved on Baker-Flex cellulose PEI plates using 0.25 M LiCl, 0.375 M Tris, 1 M formic acid; and the percentage of ATP hydrolyzed was determined from the comparison of the intensity of ADP and ATP spots with a Molecular Dynamics PhosphorImager using IMAGEQUANT software.

**Cross-Linking Assays.** Wild-type and mutant proteins dialyzed against reaction buffer 1 with 2 mM DTT were adjusted to the indicated concentrations in reaction buffer 1 containing DTT (2 mM) with or without ATP (5 mM). Assay volumes were 200 μl for 0.01 mg/ml, 120 μl for 0.02 mg/ml, 100 μl for 0.05 mg/ml, 40 μl for 0.1 mg/ml, and 10 μl for 0.5 mg/ml reactions. Reactions were incubated at 37°C for 10 min, glutaraldehyde was added (final concentration 0.1%), and cross-linking was terminated as described (11). Conditions were the same for experiments with CHAPS except that 3 mM ATP was used instead of 5 mM.

**Results.**

**The Relationship Between Hexamerization and Protein Concentration.** In buffers that approximate physiological salt concentrations, the assembly of Hsp104 into hexamers is driven by the addition of adenine nucleotides (11). In the absence of added nucleotide, Hsp104 forms hexamers if salt concentrations are low (i.e., 20 mM; ref. 10). We sought additional conditions that would promote assembly in the absence of ATP and found that increasing the protein concentration in physiological ionic strength buffers did so (Fig. 1A). When Hsp104 was cross-linked with glutaraldehyde at a protein concentration of 0.1 to 0.5 mg/ml, most of the protein migrated as a hexamer (this is apparent from counting the steps in the ladder of species obtained by cross-linking with glutaraldehyde at shorter incubation times). When Hsp104 was cross-linked at a concentration of 0.01 mg/ml, most of the protein migrated as a monomer. When ATP was present, cross-linking yielded hexamers at all of these protein concentrations. Gel filtration chromatography and multiance light scattering confirmed that Hsp104 was hexameric at high protein concentrations and existed as a combination of monomers and dimers at low protein concentrations (D. Hattendorf and S.L, unpublished results).

For simplicity, we refer to the latter state as “unassembled.”

We next asked if high protein concentrations could overcome the oligomerization defects of Hsp104 NBD2 point mutants (Fig. 1B), which fail to assemble at ATP concentrations at which the WT protein is fully assembled (10, 11). One of these, K620T, carries a threonine substitution in the conserved lysine of the P-loop motif that interacts directly with the β- and γ-phosphates of bound nucleotide in other nucleotide-binding proteins with this motif (21); the other, G619V, is a glycine to valine substitution in residue 619 (Fig. 1B). Previously, only K620T was tested at a protein concentration of 0.01 mg/ml. It was un assembled even in the presence of 5 mM ATP (10, 11). Here, for both of these mutants, some assembly was observed at a protein concentration of 0.05 mg/ml; at 0.5 mg/ml, nearly all of the protein was hexameric (Fig. 1C and data not shown). Note that proteins carrying analogous mutations in NBD1, K218T, G217V, and a new double mutant K218T:A315T with severe defects in ATPase activity assembled in the presence of ATP even at low protein concentrations (Fig. 1C and data not shown).

**The Relationship Between Hexamerization and ATP Hydrolysis in the NBD2 Mutants.** Next, we asked if overcoming the assembly defect of NBD2 mutants would also overcome their ATP hydrolysis defects. At protein concentrations of 0.01 to 0.02 mg/ml and an ATP concentration of 5 mM, the K620T and G619V proteins hydrolyzed ATP at ~1/20th the rate of WT Hsp104 (10). At
higher protein concentrations, the rate of ATP hydrolysis increased dramatically, reaching 40–80% of WT levels at 0.5 mg/ml (Fig. 2 and data not shown).

The two proteins that carried analogous P-loop substitutions in NBD1, K218T and G217V (10) failed to hydrolyze detectable amounts of ATP at any protein concentration tested (Fig. 2 and data not shown). The same was true for the double mutant K218T:A315T. Another mutant, not previously described, carried a threonine to alanine substitution adjacent to the P-loop lysine in NBD2, T621A. In the presence of ATP, this mutant assembled into hexamers, even at low protein concentrations (Fig. 1C). Its ATPase activity was lower than WT protein but did not change at any of the concentrations tested (Fig. 2). These data suggest that the increase in ATP hydrolysis with the G619V and K620T proteins at high concentrations results from overcoming their assembly defects.

**Kinetic Analysis of Mutant and WT Proteins.** To determine what parameters of ATP hydrolysis are affected by assembly, the $V_{\text{max}}$ and $K_m$ values for ATP hydrolysis were determined at different protein concentrations. At the highest and lowest concentrations, linearity was lost over the broad range of ATP concentrations required for these assays, but at protein concentrations between 0.02 and 0.1 mg/ml, ATPase activity remained linear from 500 μM to 25 mM. As determined by cross-linking, most K620T or G619V protein was in the unassembled form at 0.02 mg/ml; most was in the hexameric form at 0.1 mg/ml (data not shown). For WT Hsp104 at 0.1 and 0.02 mg/ml, the $K_m$ and $V_{\text{max}}$ values were similar (Fig. 3; Table 1). For K620T, the $V_{\text{max}}$ increased at the higher protein concentration. However, no further increase in $V_{\text{max}}$ occurred when the protein concentration was further increased from 0.1 to 0.25 mg/ml (ATPase activity remained in the linear range for K620T at 0.25 mg/ml). That is, once this mutant protein assembled, $V_{\text{max}}$ did not continue to increase as the protein concentration increased.

Because WT Hsp104 forms hexamers even at very low protein concentrations when ATP is present, we used a different experimental approach to examine the effects of assembly on ATP hydrolysis. As determined by glutaraldehyde cross-linking (Fig. 4A) and by sizing chromatography (J. R. Glover and S.L., unpublished observations), the assembly of WT Hsp104, in the presence of ATP, can be manipulated with the zwitterionic detergent CHAPS. WT Hsp104 assembles into hexamers in 2

![Image 1](82x308 to 271x733)

Fig. 1. Assembly of Hsp104 proteins. (A) Effect of high protein concentrations on the assembly of WT Hsp104 into hexamers. WT Hsp104 protein at different concentrations was incubated at 37°C with glutaraldehyde in the presence or absence of ATP for 2 or 12 min. Cross-linked proteins were separated on SDS/3.5% polyacrylamide gels and stained with silver. Intramolecular cross-links increased the mobility of monomers indicated by brackets. In the absence of ATP, most Hsp104 was unassembled when cross-linked at low protein concentrations but hexameric (indicated by asterisks) when cross-linked at high protein concentrations. In the presence of ATP, Hsp104 remained in the linear range for K620T at 0.25 mg/ml. ATP hydrolysis by the K218T:A315T and G217V mutants was negligible at all protein concentrations, but the low hydrolysis by the K620T and G619V mutants increased more than 10-fold at high protein concentrations. No change occurred in the T621A mutant, which was oligomized at all protein concentrations.

![Image 2](317x603 to 561x732)

Fig. 2. Effects of protein concentration on the ATPase activity of mutant Hsp104 proteins. ATPase assays were performed with 5 mM ATP, using the Malachite Green colorimetric assay to measure released phosphate. The same buffer conditions were used as in cross-linking studies with different concentrations of Hsp104. The endpoints of reactions were varied to keep them in the linear range of the assay and subsequently adjusted to min⁻¹. Mutant activity is shown as the % of WT activity in the same experiment; mean and standard deviations are shown. WT Hsp104 released 0.67, 0.77, 1.10, 0.89, 1.02, and 0.80 nmol of Pi min⁻¹ μg⁻¹, respectively, at 0.01, 0.02, 0.05, 0.1, 0.25, and 0.5 mg/ml. ATP hydrolysis by the K218T:A315T and G217V mutants was negligible at all protein concentrations, but the low hydrolysis by the K620T and G619V mutants increased more than 10-fold at high protein concentrations. No change occurred in the T621A mutant, which was oligomized at all protein concentrations.
mM CHAPS (below the critical micelle concentration) but not in 10 mM CHAPS (just above the critical micelle concentration). When assembly of WT Hsp104 was inhibited with CHAPS, ATP hydrolysis was reduced, albeit not as strongly as in the unassembled K620T (Fig. 4B). Notably, however, reducing the CHAPS concentration to 2 mM promoted assembly, thereby increasing the rate of hydrolysis by increasing V_max severalfold. Together, these data indicate that the rate of turnover that is most affected when Hsp104 assumes a hexameric form rather than the affinity for ATP (at least at the site, or sites, responsible for the majority of ATP hydrolysis under these conditions).

ATP Hydrolysis Is Cooperative in Hexameric Hsp104. One mechanism by which the interaction between Hsp104 subunits might increase the rate of ATP hydrolysis is through cooperativity, with ATP hydrolysis at one site increasing the likelihood of hydrolysis at another. Positive cooperativity should produce a Hill coefficient greater than 1. A series of kinetic assays were performed over a broad range of ATP concentrations (10 μM to 40 mM) at a concentration where both WT Hsp104 and K620T were hexameric. For each protein, the Hill coefficient was repeatedly determined by least squares fitting to the Michaelis–Menten equation by using the KALEIDAGRAPH program (Synergy Software). Kinetic properties of the WT protein are similar at both protein concentrations, but for the K620T mutant V_max values increased 4-fold at the higher protein concentration.

The Role of Cooperativity in Hsp104 Function. To investigate the importance of cooperative subunit interactions in vivo, we sought conditions by which it could be blocked in a biological context. The mutant K218T:A315T was the logical choice to pursue this aim. It virtually eliminated ATP hydrolysis, yet it efficiently assembled into hexamers, both on its own and in the presence of WT Hsp104 protein (by glutaraldehyde cross-linking, data not shown). A series of kinetic assays were performed in the presence of CHAPS. WT Hsp104 (0.02 mg/ml) was incubated for 16 min and K_m and V_max values were calculated as in Fig. 3. In different experiments, the V_max for ATP hydrolysis was 2- to 4-fold less in 10 mM CHAPS (hexameric, dashed line) than in 2 mM CHAPS ( unassembled), whereas K_m was changed less than 2-fold.

### Table 1. K_m and V_max values calculated for the K620T and WT proteins at two different protein concentrations

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc., mg/ml</th>
<th>K_m, mM</th>
<th>V_max, nmol min⁻¹μg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>K620T</td>
<td>0.02</td>
<td>2.3</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>2.5</td>
<td>0.25</td>
</tr>
<tr>
<td>WT</td>
<td>0.02</td>
<td>4.8</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>5.9</td>
<td>1.28</td>
</tr>
</tbody>
</table>

V_max is in nmol of P_i released per min per μg of protein.
produced by WT Hsp104 after heat shock.

growth in the absence of heat shock but blocked the increase in survival
determine survival at 25°C before (No HS) or after (HS) a 4-min heat shock at
NO HS panel. Cells were serially diluted (5-fold each step) and plated to
protein before heat shock are shown for equal numbers of cells beneath the

Hsp104 reduces it approximately 2-fold.

conclude that excess K218T:A315T blocks cooperative ATP
activity in a nearly additive manner (data not shown). Thus, we
the addition of K620T to WT Hsp104 increased the total ATPase

K218T:A315T is required to maximize the effects of this mutant
to provide thermotolerance in the absence of WT Hsp104 but

tions. However, very little functional Hsp104 is required for
thermotolerance (D. A. Parsell and S.L., unpublished observa-

K218T and WT Hsp104 at similar levels had normal levels of
has no deleterious effects on growth (20). Cells coexpressing
K218T:A315T to a fixed quantity of WT protein (0.5

ii) Effect of the K218T:A315T protein
on the biological activity of WT Hsp104. Cells had an integrated, constitutive
K218T:A315T expression plasmid or the vector control and an estradiol-

K218T:A315T to a fixed quantity of WT protein (0.5

CHAPS in the reactions to prevent oligomerization). This was
the rate of hydrolysis is similar to that obtained by including
chap in the reactions to prevent oligomerization). This was
not due to competition for substrate, as ATP was in excess, nor
was it due to the change in protein density because (i) the
addition of BSA in place of K218T:A315T did not inhibit
hydrolysis by Hsp104, (ii) the addition of WT Hsp104 in place of
K218T:A315T increased hydrolysis in a linear manner, and (iii)
the addition of K620T to WT Hsp104 increased the total ATPase
activity in a nearly additive manner (data not shown). Thus, we
conclude that excess K218T:A315T blocks cooperative ATP
hydrolysis by WT Hsp104. At its maximum effect, this block in
cooperativity is not so strong as to eliminate hydrolysis; it simply
reduces it approximately 2-fold.

Next, we examined the effects of excess K218T:K315T on WT
Hsp104 in vivo. We have previously shown that K218T is unable
to provide thermotolerance in the absence of WT Hsp104 but
has no deleterious effects on growth (20). Cells coexpressing
K218T and WT Hsp104 at similar levels had normal levels of
thermotolerance (D. A. Parsell and S.L., unpublished observa-
tions). However, very little functional Hsp104 is required for
thermotolerance (28, 29). Thus, those Hsp104 hexamers that do
not contain sufficient K218T:A315T to block cooperative ATP
hydrolysis by WT Hsp104 could well be sufficient for thermo-
tolerance. In light of our observation that a 5-fold excess
of K218T:A315T is required to maximize the effects of this mutant
on the ATPase activity of WT Hsp104 in vitro (Fig. 6A), we
engineered the expression of K218T:A315T such that the
K218T:A315T/WT ratio would be ~5 in vivo. In this case,
thermotolerance was reduced dramatically. In the absence of
heat shock, high levels of the K218T:A315T protein had no
adverse effect on growth in either cells expressing WT Hsp104
or cells carrying an HSP104 deletion (Fig. 6B, NO HS; data not
shown). Moreover, the K218T:A315T mutant did not reduce the
low level of residual thermotolerance that is present in hsp104
deletion cells after a conditioning preheat treatment (data not
shown). Because the K218T:A315T mutant had no deleterious
effects on its own, we suggest that the decrease in thermotoler-
ance it produces in the presence of WT Hsp104 is due to the
ability of this mutant to alter the activity of the WT protein in
mixed oligomers.

The K620T mutant was also assayed in vivo. In the absence of
WT Hsp104, overexpressing K620T had no deleterious effects on
growth. However, overexpression of K620T in the absence of
WT Hsp104 caused some growth inhibition at normal tempera-
tures (data not shown), preventing testing of the effect of
K620T on the thermotolerance function of WT Hsp104. None-
thless, it provides independent evidence of the importance of
subunit interactions in regulating Hsp104 function in vivo.

Discussion
We have shown that the activities of Hsp104 are strongly
influenced by subunit interactions, both in vitro and in vivo. In
experiments where the hexameric form of WT Hsp104 was
controlled by the detergent CHAPS or the hexameric form of
NBD2 mutants was controlled by protein concentration, ATP
hydrolysis was higher in the assembled state than in the nonas-
sembled state by about 4-fold. In both cases, this was primarily
due to an increase in V_{max}. We have also determined that
hydrolysis is cooperative in Hsp104, thereby identifying a con-
tributing factor for increased hydrolysis in the assembled state.
Our data further suggest that cooperativity and subunit in-
teractions are vital to the thermotolerance function of Hsp104
in vivo.

Earlier work had indicated functional distinctions between the
two NBDs of Hsp104 (10, 11); NBD1 mutations virtually elimi-
nate hydrolysis without affecting assembly, whereas analogous
mutations in NBD2 severely affect assembly while reducing
hydrolysis ~10-fold. We now find that high protein concentra-
ations overcome the assembly defects of NBD2 mutants and at the
same time increase the rate of ATP hydrolysis to ~40–80% that
of the WT. Together with earlier data, these observations argue
strongly that NBD1 is responsible for most of the ATPase
activity of the protein, whereas nucleotide binding or hydrolysis
at NBD2 regulates structural parameters that affect NBD1
activity (for example, in response to substrate and cofactor
interactions). It remains possible that both sites contribute more
equally to hydrolysis. If so, the ATPase activities of the two
domains must be coupled in such a way that the basal activity
of NBD2 strongly depends on the activity of NBD1, but the basal
activity of NBD1 is relatively independent of NBD2 activity
when the protein is assembled.

If most ATP hydrolysis occurs at NBD1, then the Hill
coefficient of ~2 likely reflects cooperative hydrolysis be-
tween NBD1 subunits in the hexamer rather than between
NBD1 and NBD2. This idea is further supported by the
allosteric interactions we observed by inhibiting WT Hsp104
ATPase activity in mixed oligomers, an effect that has also
been observed for the related ClpA protein (22). Furthermore,
a residue in the C terminus that is highly conserved among all
HSP100 proteins was shown to interact with a conserved
P-loop residue of an adjacent subunit in oligomers of HslU,
another member of the HSP100 protein family (30). And in
separate work, we have found that the affinity of NBD2 for
ATP is 100-fold greater than that of NBD1 (D. Hattendorf
and S.L., unpublished results). Most of the kinetic parameters
of ATP hydrolysis examined here reflect the effects of ATP binding to the lower affinity site, NBD1.

Unlike HslU, which has only one NBD and a C-terminal region, Hsp104 has two NBDs flanked by N-terminal, middle, and C-terminal regions. Thus, a large number of interactions between domains and subunits is possible. Indeed, we have found that the rate of ATP hydrolysis increases substantially when polypeptides are bound to the C terminus of Hsp104 and that the middle region of Hsp104 appears to play an important role in mediating changes in NBD1 activity in response to C-terminal interactions (A. Cashikar, E.C.S., D.M.W., and S.L., unpublished data). This middle region of Hsp104 and other HSP100 proteins that function in thermotolerance is long, whereas that of the HSP100/Cip proteins that function in proteolysis is much shorter or absent; thus, the middle region’s effects on the ATPase reaction cycle may distinguish these functions. In separate work, we have found that the rate of ATP hydrolysis in assembled Hsp104 is increased in low salt buffers (10). In that case, the increase in hydrolysis specifically reflected a change in the $K_m$ measured by ATP hydrolysis. In contrast, the effects of oligomerization reported here specifically affect $V_{max}$. Taken together, this work suggests that the two NBDs of Hsp104 work together in a complex cycle that involves multiple ATP binding and hydrolysis states, which may be influenced by many interactions within the hexamer and with substrates.

In addition to demonstrating that cooperativity and oligomeric contacts influence the parameters of ATP hydrolysis by Hsp104, we have established that these contacts profoundly influence the function of the protein in living cells. Unlike many other chaperones, WT Hsp104 is not toxic to growth even when it is overexpressed at very high concentrations (28, 29). The fact that K620T slightly inhibits growth at normal temperatures, but only in the presence of WT protein, suggests correct subunit interactions are normally required to control the biological activities of Hsp104. Further, when the K218T:A315T mutant was expressed in the presence of WT Hsp104 at a ratio equivalent to that which maximally inhibits WT Hsp104 ATP hydrolysis in vitro, it strongly inhibited the thermotolerance function of WT Hsp104 in vivo. Yet, the K218T:A315T mutant had no toxic effects on its own. The observations reported here provide a biological confirmation of earlier biochemical suggestions that the two NBDs of Hsp104 have different functional roles. They also indicate that correct subunit interactions in the hexameric form are vital for thermotolerance function. This was by no means a foregone conclusion. K218T:A315T reduced the free-running ATPase activity of WT Hsp104 in mixed oligomers by only ~2-fold, and we have previously shown that a very small quantity of Hsp104 is sufficient for thermotolerance (28). Yet, even larger quantities of WT Hsp104 are insufficient for thermotolerance when cooperative subunit interactions are perturbed by the induction of K218T:A315T. The conditional expression of this protein will provide an important tool for the analysis of Hsp104 function in the maintenance of yeast prions and the aggregation-prone human disease proteins such as huntingtin.

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