The Prion Curing Agent Guanidinium Chloride Specifically Inhibits ATP Hydrolysis by Hsp104*

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The molecular chaperone Hsp104 from Saccharomyces cerevisiae dissolves protein aggregates in the cell and is thus of crucial importance for the thermotolerance of yeast. In addition to this disaggregate activity, Hsp104 has a key function in yeast prion propagation, as Hsp104 was found to be essential for the maintenance of the associated phenotypes. In vivo data suggest that Hsp104 function is affected by guanidinium chloride. Adding small amounts of this compound to yeast medium causes curing of the prions: cells lose their prion-related phenotype. Guanidinium chloride was also found to impair heat shock resistance. Here, we present a detailed in vitro analysis showing that guanidinium chloride is an uncompetitive inhibitor of Hsp104. Micromolar concentrations of this agent reduce the ATPase activity of Hsp104 to ~35% of its normal activity. This inhibition is not related to the denaturing properties of this compound, because Hsp104 was not affected by urea. Guanidinium ions selectively bind to the nucleotide-bound, hexameric state of the molecular chaperone. Thus, they increase the affinity of Hsp104 for adenine nucleotides and promote the nucleotide-dependent oligomerization of the chaperone. Our findings strongly suggest that guanidinium chloride causes curing of yeast prions by perturbing the ATPase of Hsp104, which is essential for both prion propagation and thermostolerance.

Aggregation of proteins is a common phenomenon in living cells. Protein aggregates can, for example, form during stress conditions such as heat shock or the biogenesis of labile proteins. The molecular chaperone Hsp104 from Saccharomyces cerevisiae is crucial for the disaggregation of protein aggregates and thus constitutes a major factor for thermostolerance in yeast (1, 2). Disaggregation appears to be a complex process requiring the assistance of several other chaperones, including the Hsp70 system (3). However, the molecular details of this reaction are poorly understood.

In addition to its involvement in thermostolerance, Hsp104 plays a key role in the propagation and inheritance of the yeast prions [PSI+] and [URE3] (4–7). Although the underlying mechanism is still unknown, it is likely that in both cases, thermostolerance and prion propagation, analogous interactions with non-soluble protein aggregates occur (8). When the expression level of Hsp104 is decreased, both the recovery from heat stress and prion replication are affected (4, 7, 9, 10). Remarkably, the addition of small amounts of the chaotropic salt guanidinium chloride (GdmCl) to the growth medium also leads to a loss of prion propagation, a phenomenon termed curing (11). Curing by GdmCl has been observed for all yeast prions tested to date (9, 10, 12–14). Although GdmCl seems to inhibit the replication of the prions (15, 16), several lines of evidence suggest that it does not target the prion proteins themselves but Hsp104. First, GdmCl blocks the disaggregase activity of Hsp104 in vivo and reduces the thermostolerance of yeast (17, 18). Second, both GdmCl-treated and Hsp104-deficient yeast cells have similar phenotypes in respect to prion propagation (17, 18). A recent report showed that, in yeast strains with a point mutation in Hsp104 (D184S), prion phenotypes can no longer be cured by GdmCl (19). Finally, Glover and Lindquist noted that low residual concentrations of GdmCl during re-activation of chemically denatured luciferase inhibit ATP hydrolysis by Hsp104 (3).

Hsp104 and its prokaryotic homologue, ClpB, belong to the Hsp100/Clp protein family (20), whose members possess characteristic nucleotide-binding domains of the AAA-ATPase (ATPases associated with a variety of cellular activities) type. As a general feature, AAA-ATPases promote structural changes in bound substrates by ATP-driven conformational movements (21, 22). Hsp104 has two nucleotide-binding domains, NBD1 and NBD2, and forms ring-shaped hexamers (23). The oligomerization equilibrium is highly dynamic and is influenced by numerous parameters, including not only protein concentration but also ionic strength and nucleotides. In the absence of nucleotides, Hsp104 is a mixture of monomeric and hexameric species, but other species such as dimers may exist as well (24, 25). Binding of ADP or ATP to NBD2 shifts this distribution toward the hexameric form (23). High salt concentrations, on the other hand, stabilize monomeric Hsp104 (25). Importantly, ATP turnover by Hsp104 seems to be highly dependent on its quaternary structure. Studies by Lindquist and co-workers showed that mutants of Hsp104 that are defective in oligomerization possess virtually no ATPase activity (24). While NBD2 is involved in Hsp104 assembly, NBD1 seems to account for most of the ATP hydrolysis of Hsp104 under steady-state conditions (25).

Here, we present a detailed in vitro analysis showing that GdmCl is a competitive inhibitor of Hsp104. GdmCl binds only to the nucleotide-bound form of Hsp104 and increases the affinity to nucleotides while reducing $k_{\text{cat}}$ at the same time. Our

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§ The abbreviations used are: GdmCl, guanidinium chloride; Gdm+, guanidinium ion; ITC, isothermal titration calorimetry; NBD, nucleotide-binding domain; SLS, static light scattering; AMPNP, adenosine 5’-(β,γ-imino)triphosphate.

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results confirm a direct link between prion curing and inactivation of Hsp104.

**EXPERIMENTAL PROCEDURES**

**Cloning of Hsp104 Gene from S. cerevisiae**—The gene encoding Hsp104 was isolated from yeast strain W303 using PCR amplification with appropriate primers (forward, 5'-AAA GAT CAT GCA CCA AAC GCA ATT TAC AGA AAG-3'; reverse, 5'-AAA AGG ATC CCT TTA CTA CTT CAT CA-3'). Amplified DNA fragments were cloned into the vector pQE70 (Qiagen, Hilden, Germany) using restriction sites for SpaI and BamHI. The insert contained the authentic stop codon, resulting in a protein that lacks the C-terminal His8 tag encoded by the vector. Successful cloning was verified by DNA sequencing.

**Production of Recombinant Hsp104**—Plasmids carrying the Hsp104 genes were transformed into E. coli BL21 (CodonPlus DE3) RIL (Stratagene). For protein production, 6 liters of Luria Bertani medium containing 100 mg/l ampicillin were inoculated with an overnight culture. For protein production, 6 liters of Luria Bertani medium containing 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and cells were harvested 3 h later. Purification of Hsp104 was carried out as described previously (28). The concentration of Hsp104 was determined using an extinction coefficient of ε270 nm = 31,900 M⁻¹ cm⁻¹. Molar concentrations of Hsp104 refer to the monomeric species.

**ATPase Assays**—ATP hydrolysis by Hsp104 was monitored using a coupled enzymatic assay in combination with an ATP-regenerating system (29). This setup avoids some of the problems arising from the fact that ADP binds to Hsp104 more strongly than ATP does and, thus, is a potent competitive inhibitor (25). Briefly, the ATP hydrolyzed by Hsp104 is regenerated by pyruvate kinase and phosphoenolpyruvate (PEP), resulting in the formation of pyruvate. In a second step, pyruvate is reduced to lactate by lactate dehydrogenase and NADH, a reaction that can be followed by the decrease in absorbance at 340 nm. The assay mixture contained 2 mM phosphoenolpyruvate, 0.2 mM NADH, 2 units/ml pyruvate kinase, and 10 units/ml L-lactate dehydrogenase (both from rabbit muscle). All enzymes and reagents used in the assay were from Roche Applied Science. Assays were carried out in thermostatted 120-µl cuvettes at 30 °C using a Cary50 spectrophotometer (Varian, Palo Alto, CA). Assays were performed in assay buffer (50 mM Hepes/KOH, pH 7.5, 150 mM KCl, and 10 mM MgCl₂) containing 5 mM ATP and 0.5 µM Hsp104 unless indicated otherwise. The rate of ATP hydrolysis, V, was determined from the slope dA405 nm/dt using a molar absorbance coefficient for NADH of ε340 nm = 6,200 M⁻¹ cm⁻¹. For each data point, four independent measurements were carried out to calculate the mean value. The corresponding S.D. values are shown as error bars in the respective figures. To simplify the comparison of rates obtained for different enzyme concentrations, we use the apparent rate constant of hydrolysis, k_app = V/[Hsp104], to quantify ATP turnover.

To evaluate the dependence of ATP hydrolysis on ATP concentration, values of V were fitted to the model of Michaelis-Menten, shown in Equation 1:

\[
V = \frac{k_{cat}[E]_0}{[ATP] + K_m} \]  \hspace{1cm} (Eq. 1)

in which k_cat is the rate constant of turnover under saturation conditions, [E]₀ is the total enzyme concentration, and Kₘ is the Michaelis constant.

**Static Light Scattering (SLS)**—SLS measurements of Hsp104 were carried out in assay buffer at 20 °C using a miniDAWN Tristar detector (Wyatt Tec., Santa Barbara) equipped with a flow cell. Samples containing 0.5-2.5 mM Hsp104, 5 mM GdmCl, and 5 mM ATP in various combinations were injected.

**Isothermal Titration Calorimetry**—Isothermal titration calorimetry (ITC) was performed using a MicroCal VP-ITC (MicroCal Inc., Northampton). All experiments were performed at 30 °C using assay buffer. For the binding of GdmCl to Hsp104 or Hsp104-nucleotide complexes, titrations were carried out using 14.1 mM GdmCl in the injection syringe and an injection volume of 5 µl. The protein concentration in the cell was 30 µM. For the binding of ADP to Hsp104 in presence of GdmCl, the injection syringe was filled with a solution of 2.82 mM nucleotide. The protein concentration in the cell was 25 µM. Identical concentrations of GdmCl were added to the syringe and the cell. Data analysis was performed with the Origin software package (Origin Lab, Northampton, MA).

**Analytical Ultracentrifugation**—Analytical ultracentrifugation was performed on a Beckman XL-I analytical ultracentrifuge equipped with UV/Vis-optics and interference optics. Rotation speed for sedimentation runs was 50,000 rpm, and the protein was detected based on the absorbance at 280 nm. Scans were performed every 7 min. Aluminum centerpieces were used that contained the buffer and the protein solution (10 µM) in separate chambers. Measurements were carried out in 50 mM Hepes/KOH, pH 7.5, 800 mM KCl, and 10 mM MgCl₂ at 25 °C. To examine the influence of GdmCl on hexamerization, samples additionally contained 30 µM ADP and/or 10 mM GdmCl. The distance of the sedimentation boundary from the center of the rotor, r, was determined for every scan, and the sedimentation coefficient, s, was calculated using Equation 2,

\[
ln r = \omega^2 (r - r_0)
\]

in which r represents the position of the boundary at the start of the experiment, t₀ is the angular velocity of the rotor, and t is the time of the scan.

**RESULTS**

**Low Concentrations of GdmCl Inhibit ATP Hydrolysis by Hsp104**—We first determined the influence of GdmCl on ATP hydrolysis by Hsp104 under steady-state conditions. Because of the strong inhibitory effect of ADP on Hsp104 (see below), we employed an assay that contains an ATP-regenerating system consisting of pyruvate kinase and lactate dehydrogenase (see “Experimental Procedures”). The data in Fig. 1 demonstrate that GdmCl markedly reduces the ATPase activity of Hsp104 at concentrations as low as 30 µM. However, even at saturating concentrations of GdmCl (up to 100 mM; data not shown), ATP hydrolysis by Hsp104 is not abolished completely but only drops to ~50% of the initial value. Because GdmCl may simply affect Hsp104 because of its denaturing properties, we performed assays using urea instead of GdmCl. In contrast to GdmCl, urea had no effect on ATP hydrolysis even at concentrations exceeding 10 mM (Fig. 1). This is in agreement with findings that, unlike GdmCl, urea cannot cure yeast prion phenotypes (28). Thus, we can rule out that GdmCl interferes with the ATPase activity of the chaperone by simply denaturing Hsp104. Furthermore, we conclude that the guanidinium ions (Gdm⁺) and not the chloride ions are responsible for the inhibition of Hsp104, because the assay buffer contains a salt background of 150 mM KCl. To verify the specificity of this effect, we examined whether GdmCl could inhibit other ATP-hydrolyzing, oligomeric chaperones such as GroEL and Hsp90. None of these enzymes exhibited sensitivity to GdmCl (data not shown). Thus, we can exclude that Gdm⁺ inhibits the enzymes.
in the regenerative ATPase assay or changes the catalytic properties of ATPases in general.

**GdmCl Does Not Affect Hexamerization of Hsp104**—It has been shown previously that the rate of ATP hydrolysis is highly dependent on the oligomeric state of Hsp104. The assembly into hexamers strongly increases the ATPase activity (24). Thus, GdmCl could indirectly reduce the apparent rate constant of ATP turnover, \( k_{\text{app}} \), by stabilizing the less active monomers. To distinguish between direct inhibition of ATP hydrolysis and indirect effects on oligomerization, we used SLS to determine whether GdmCl changes the apparent molecular weight of Hsp104. Samples containing Hsp104, GdmCl, and ADP in various combinations were loaded on the sample loop of an high pressure liquid chromatography system and then injected into a SLS detector equipped with a flow cell. Fig. 2A shows a representative SLS trace for Hsp104 at a concentration of 1 μM. Upon the addition of 5 mM ADP, the scattering signal increases by 120% (cf. traces 1 and 3), indicating an increase in the apparent molecular weight due to the ADP-induced hexamerization of the chaperone. Because the samples contained some particles with high scattering intensity, we were not able to accurately calculate the apparent molecular weight. However, Fig. 2A clearly shows that ADP promotes the oligomerization of Hsp104 and that our experimental setup is suited to detect changes in the oligomeric state of Hsp104.

When samples containing GdmCl were injected, we did not observe significant changes in the apparent molecular weight of the protein in comparison to the measurements in the absence of GdmCl (Fig. 2A). Thus, GdmCl does not seem to disrupt the oligomeric state of the chaperone, either in the presence or the absence of ADP.

Support for this conclusion comes from experiments in which we determined the influence of protein concentration on the GdmCl effect. When the concentration of Hsp104 is increased from 0.5 to 5 μM, the apparent rate of hydrolysis, \( k_{\text{app}} \), increases slightly from 42 to 57 min\(^{-1}\) (Fig. 2B, black bars). In the presence of 5 mM GdmCl, the activity of Hsp104 drops by ~50%, irrespective of the protein concentration used in the assay (Fig. 2B, white bars). This result argues against GdmCl being an inhibitor of oligomerization. Otherwise, its inhibitory effect should be less pronounced at high protein concentrations, where the hexameric state of Hsp104 is more stable.

**Binding of GdmCl to Hsp104 Is Nucleotide-dependent**—As demonstrated above, GdmCl is not an inhibitor of oligomerization but specifically reduces ATP turnover. To obtain a more detailed picture, we directly studied the interaction between GdmCl and Hsp104 using ITC.

When GdmCl was titrated to Hsp104 in the absence of ADP, no binding signal could be monitored (Fig. 3A). Thus, GdmCl does not seem to bind to nucleotide-free Hsp104. However, when we carried out the titration with 5 mM ADP present in both injection syringe and sample cell, we did observe binding of GdmCl to Hsp104 (Fig. 3B). Using a model with one binding site per monomer, the analysis of the ITC data yielded an apparent \( K_p \) of 600 μM. In the absence of Hsp104, no signal could be observed (data not shown). We can therefore rule out that the signal reflects the formation of a complex between GdmCl and ADP. Further experiments showed that GdmCl binds to Hsp104-ADP·S and Hsp104-AMPPNP with similar affinity (data not shown). In summary, our results suggest that GdmCl interacts only with nucleotide-bound Hsp104, although the type of adenosine nucleotide seems to be less important.

**GdmCl Increases the Affinity of Hsp104 for Nucleotides**—Because GdmCl only binds to Hsp104 in the presence of nucleotide, thermodynamics predicts that GdmCl, in turn, should promote nucleotide binding. Thus, we carried out a series of ITC experiments in which we titrated Hsp104 with an ADP stock solution in the presence of increasing concentrations of GdmCl. From the binding curves, we calculated the corresponding \( K_p \) for ADP. The signal-to-noise ratio of the ITC data was not sufficient to discriminate between binding to NBD1 and NBD2, respectively, and we fitted the data to a simplified model with just one (average) binding site (Fig. 4).

It is apparent that increasing concentrations of GdmCl enhance the binding of ADP to Hsp104. In the absence of GdmCl, ADP binds with an apparent \( K_p \) of ~50 μM. In the presence of 10 mM GdmCl, this value decreases to ~25 μM. The increased affinity toward ADP is mainly due to an increase in binding enthalpy, \( \Delta H \), in the presence of GdmCl (data not shown). At saturating concentrations of GdmCl, binding of ADP is 2.5 times tighter than in the absence of GdmCl.

**GdmCl Stimulates the Assembly of the Hsp104 Hexamer**—Our ITC measurements show that the affinity of Hsp104 for nucleotides is increased in the presence of GdmCl. Because

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**Fig. 2. GdmCl does not dissociate hexameric Hsp104.** A, traces represent the signal monitored by static light scattering. An increase in signal intensity reflects an increase in the apparent molecular weight of the protein (1 μM). Measurements were performed at room temperature in assay buffer. Some of the samples contained 5 mM ADP and/or 5 mM GdmCl as indicated. w/o, without additions. B, ATPase assays were carried out at 30 °C in buffer containing 5 mM ATP in the absence (black bars) or presence of 5 mM GdmCl (white bars).

**Fig. 3. Binding of GdmCl to Hsp104 is nucleotide-dependent.** Binding of GdmCl to Hsp104 in the absence of nucleotide (A) and the presence of 2 mM ADP (B) was measured using isothermal titration calorimetry. A stock solution of GdmCl (14.1 mM) was titrated to a solution of Hsp104 (30 μM) in assay buffer at 30 °C, and the associated heat change was monitored (upper panels). After integration of the injection peaks, the resulting binding curves (lower panels) were analyzed assuming one binding site per Hsp104 monomer.
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nucleotide binding induces hexamerization of Hsp104, we next asked whether GdmCl could also promote the nucleotide-dependent hexamerization of Hsp104. The SLS traces recorded in the presence of ADP seem to indicate that this is not the case (cf. Fig. 2A). However, these measurements were carried out under conditions where Hsp104 was completely hexameric, and a stimulatory effect of GdmCl would have gone unnoticed.

Thus, we employed analytical ultracentrifugation to search for conditions under which Hsp104 is dissociated even in the presence of ADP. Our screen showed that, at 800 mM KCl, the sedimentation coefficient of Hsp104 hardly increases upon the addition of 30 μM ADP (8.7 versus 8 S), indicating that the protein can no longer associate under these conditions (compare Fig. 5C). This is in agreement with a study reporting that, at a high salt concentration, the hexameric state of Hsp104 is destabilized (25). The sedimentation profile of a protein sample containing 800 mM KCl and 30 μM ADP is shown in Fig. 5A. The lines represent absorbance scans recorded at increasing times of sedimentation. Upon the addition of 10 mM GdmCl to the buffer (Fig. 5B), the sedimentation of the protein is significantly faster (s = 13.3 S), suggesting that the hexameric species becomes more populated. This stimulatory effect of GdmCl on Hsp104 assembly is even more evident when the migration of the sedimentation boundary is plotted as a function of time (Fig. 5C). The slope of this graph is a measure for the sedimentation velocity of the protein. Clearly, sedimentation is faster in the presence of GdmCl, indicating that Hsp104 increases in size. Importantly, we only observed this effect of GdmCl when ADP was present in the sample (data not shown). These results demonstrate that GdmCl can indeed stimulate the association of Hsp104 under conditions where nucleotide-dependent hexamerization is energetically less favorable.

GdmCl: An Uncompetitive Inhibitor of Hsp104—To further characterize the inhibitory effect of GdmCl on ATP hydrolysis, we examined the dependence of the turnover number on ATP concentration, both in the presence and the absence of GdmCl.

In the absence of GdmCl, a plot of ATP turnover versus ATP concentration shows a Michaelis-Menten-like profile with an apparent $K_m$ of $-11$ mM (Fig. 6A). The low affinity of Hsp104 toward ATP has been reported before, although the values of $K_m$ and $k_{cat}$ seem to be highly dependent on the experimental conditions, especially on pH and ionic strength (29). Although $K_m$ is only a crude measure for substrate affinity, it becomes obvious why using an ATP regenerating system is crucial in the case of Hsp104. ATP binding is stronger by at least two orders of magnitude (Fig. 4), and, therefore, ADP is a very potent competitive inhibitor. Above 25 mM ATP, the regenerative ATPase assay system became less reliable, and we were thus not able to obtain accurate data at higher ATP concentrations. The maximum turnover number, $k_{cat}$, was determined to be in the range of 70 min$^{-1}$, in good agreement with previous studies (24, 29). However, the ATPase activity of Hsp104 under these conditions is lower than the numbers shown in Fig. 1. This is due to an increase in MgCl2 concentration from 10 to 25 mM (data not shown) and may be explained by the pronounced salt sensitivity of Hsp104 (29).

In the presence of 5 mM GdmCl, the kinetics of hydrolysis again follow a Michaelis-Menten-like behavior, but the respective parameters $K_m$ and $k_{cat}$ are changed (Fig. 6A). At high ATP concentrations, the turnover number is 27 min$^{-1}$, only 35% of the value determined in the absence of GdmCl. At the same time, the $K_m$ for ATP is decreased to 4 mM, indicating that ATP binds more tightly in the presence of GdmCl. This finding is consistent with the effect of GdmCl on ADP binding that we observed using ITC (Fig. 4). The effect of GdmCl is best described by the model of uncompetitive inhibition in which ATP and the inhibitory enzyme-substrate complex binds to the enzyme-substrate complex (here, Hsp104-ATP) and renders it less active (30). Because the turnover of the enzyme-substrate complex is slowed down (or even abolished), $k_{cat}$ will be decreased in the presence of the inhibitor. At the same time, the inhibitor stabilizes the enzyme-substrate complex, resulting in a decrease of $K_m$. This type of inhibition can be easily identified: It gives rise to a series of parallel lines in a Lineweaver-Burk plot, because $K_m$ and $k_{cat}$ are reduced to the same extent. This is exactly what we observe for GdmCl (Fig. 6B). In the presence of 5 mM GdmCl, both $K_m$ and $k_{cat}$ decrease 2.5-fold.

DISCUSSION

It is well established that the presence of 1–5 mM GdmCl in the growth medium can cure yeast prion phenotypes, presumably by the inhibition of Hsp104 (9–12, 14). Our results show that, in vitro, these low concentrations of GdmCl indeed change the enzymatic properties of Hsp104, thereby providing a strong link between curing and Hsp104 inactivation. Our data also demonstrate that inactivation is not related to the denaturing properties of GdmCl, because 100 times higher amounts of urea have no effect on ATP hydrolysis. This is in perfect agreement with in vivo experiments showing that urea lacks the curing potency of GdmCl (28).

Binding of GdmCl to Hsp104-Nucleotide Complexes—Our ITC experiments demonstrate that binding of GdmCl to Hsp104 is dependent on nucleotides; no binding was observed in the absence of ATP. Thus, either the nucleotide directly contacts GdmCl, or the binding of GdmCl requires a conformational change in Hsp104 that is induced by nucleotides. GdmCl as a cation could, for example, interact with the negatively charged phosphate groups of the bound nucleotide. This, in turn, could slow down ATP hydrolysis, for instance by masking the γ-phosphate group from catalytic residues or the attacking water molecule. However, residues from the protein must participate in the binding of GdmCl as well because free ADP did not form a complex with GdmCl, and other ATPases, such as GroEL and Hsp90, were not affected by GdmCl.

GdmCl: An Uncompetitive Inhibitor of Hsp104—The effect of GdmCl on ATP hydrolysis by Hsp104 is adequately described by the model of uncompetitive inhibition, which requires the following: (i) that GdmCl binds to the enzyme-substrate complex, Hsp104-ATP, but not to the free enzyme; and (ii) that both $K_m$ and $k_{cat}$ are reduced to the same extent. The inhibition by GdmCl is only
Hsp104/ADP (off rate of ADP by a factor of 2.5. This would account for both.

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Fig. 5. GdmCl can stimulate hexamerization of Hsp104. Sedimentation velocity of Hsp104 (10 μM) was measured in an analytical ultracentrifuge at 25 °C in 50 mM Hepes/KOH, pH 7.5, 800 mM KCl, and 10 mM MgCl₂. A, sedimentation profile for Hsp104 in the presence of 30 μM ADP. The lines represent absorbance scans at increasing times of sedimentation. B, sedimentation profile for Hsp104 in the presence of 30 μM ADP and 10 mM GdmCl. C, the distance of the sedimentation boundary from the rotor center, r, is plotted versus the time, t, for Hsp104 alone (○), Hsp104/ADP (□), and Hsp104/ADP/GdmCl (▼). The slope dr/dt reflects the sedimentation velocity.

Partial, i.e. the ternary enzyme-substrate-inhibitor complex, Hsp104-ATP-Gdm⁺, can still form the product, although more slowly than Hsp104-ATP. In agreement with our experimental findings, the model also predicts that the inhibitory effect becomes more pronounced at a high ATP concentration (compare Fig. 6A), because the species the inhibitor binds to, Hsp104-ATP, is more populated under these conditions. It is not known which step is rate-limiting in ATP hydrolysis under steady-state conditions. But if turnover is governed by product dissociation, GdmCl may simply decrease the off rate of ADP by a factor of 2.5. This would account for both the decreased Kforge we measured with ITC and the smaller kcat in the hydrolysis experiments.

Furthermore, Gdm⁺ may not only change the rate of hydrolysis but also the molecular context of this reaction. In the case of the GroE chaperone, (productive) ATP hydrolysis occurs in a quantized manner, i.e. the seven ATP molecules bound to one ring are hydrolyzed simultaneously (31). Any compound that could affect this cooperativity would render GroE non-functional although the effect on overall ATP turnover may be small. Furthermore, one could envision that Gdm⁺ blocks the transmission of the energy provided by ATP hydrolysis to the site on Hsp104 where the “work” is actually done, whatever this work may be on a molecular level. These possibilities are, of course, not mutually exclusive.

Molecular Mechanism of Prion Curing in Yeast—Interestingly, ATP hydrolysis of Hsp104 is not completely abolished in the presence of GdmCl. Under saturating conditions it is reduced to 35% of its normal level. In vivo data, on the other hand, suggest that Hsp104 function is lost entirely when yeast grows in medium containing GdmCl, because its phenotype resembles that of an hsp104 knockout. A closer look at the time dependence of curing in vivo may provide an explanation for this seeming contradiction. Wegzyn et al. report that, although growing [PSI⁺] cells in medium containing GdmCl causes curing of the prion phenotype, this process is significantly slower than curing by removal or inactivation of Hsp104 (32). Whereas in the first case [psi⁻] cells only appear after at least 3–5 generations, curing in the second case already starts after two generations. The authors interpret these differences in curing kinetics by suggesting that either the mechanism of curing by GdmCl is independent of Hsp104 or that GdmCl does not completely inactivate Hsp104. Our results are in agreement with the latter possibility; we find that Gdm⁺ only partially inhibits the ATPase activity of Hsp104. Additional support comes from experiments in which the cellular level of Hsp104 was reduced. For curing to occur, it was sufficient to decrease the expression of the chaperone to ~25% of the normal level (32).

Over the past years, a wealth of data has been accumulated both in vivo and in vitro, showing convincingly that destroying the capability of Hsp104 to hydrolyze ATP results in a loss of function (29, 33). Most of these studies have been carried out with mutants in which conserved lysine residues in NBD1 (K218T) and/or NBD2 (K620T) have been replaced by threonine. These mutations strongly reduce the capability of the affected domain to bind or hydrolyze nucleotides (29). Both mutations reduce the thermotolerance of yeast (20) and can cause curing of the [PSI⁺] phenotype (16, 24). Our results now provide evidence that, more specifically, the rate of hydrolysis of ATP is important for the biological activity of Hsp104. In the presence of GdmCl, nucleotide binding to Hsp104 is even enhanced. Nevertheless, its chaperone function in vivo is severely compromised. Hattendorf and Lindquist have shown that, under steady-state conditions, NBD1 accounts for most of the ATPase activity displayed by Hsp104 (25). Thus, we assume that Gdm⁺ slows down ATP turnover in NBD1, which, in turn, decreases the biological activity of Hsp104.

Eaglestone et al. have put forward a conclusive model in which GdmCl prevents the Hsp104-mediated formation of new prion seeds and thus blocks further prion replication (15). The prion phenotype will eventually be lost, because the existing seeds become diluted in the following cell divisions. Although we currently do not understand the molecular mechanism of
seed formation by Hsp104, our findings show that ATP turnover is essential in this process.

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