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The ATPase Activity of Hsp104, Effects of Environmental Conditions and Mutations*

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Hsp104 is crucial for stress tolerance in *Saccharomyces cerevisiae*, and both of its nucleotide-binding domains (NBD1 and NBD2) are required. Here, we characterize the ATPase activity and oligomerization properties of wild-type (WT) Hsp104 and of NBD mutants. In physiological ionic strength buffers (pH 7.5, 37 °C) WT Hsp104 exhibits Michaelis-Menten kinetics between 0.5 and 25 mM ATP (K_m ~5 mM, V_{max} ~2 nmol min^{-1} μg^{-1}). ATPase activity is strongly influenced by factors that vary with cell stress (*e.g.* temperature, pH, and ADP). Mutations in the P-loop of NBD1 (G217V or K218T) severely reduce ATP hydrolysis but have little effect on oligomerization. Analogous mutations in NBD2 (G619V or K620T) have smaller effects on ATPase activity but impair oligomerization. The opposite relationship was reported for another member of the HSP100 protein family, the *Escherichia coli* ClpA protein, in studies employing lower ionic strength buffers. In such buffers, the K_m of WT Hsp104 for ATP hydrolysis decreased 10-fold and its stability under stress conditions increased, but the effects of the NBD mutations on ATPase activity and oligomerization remained opposite to those of ClpA. Either the functions of the two NBDs in ClpA and Hsp104 have been reversed or both contribute to ATP hydrolysis and oligomerization in a complex manner that can be idiosyncratically affected by such mutations.

HSP100/Clp proteins are found in eubacteria, fungi, plants, and animals including man. They function in a wide variety of biological activities such as stress tolerance, proteolysis, DNA transposition, and gene regulation. These diverse activities involve a common biochemical mechanism, disassembling quaternary protein structures and aggregates (1). For example, the ClpA protein of *Escherichia coli* regulates the activity of the ClpP protease, promoting the hydrolysis of specific substrates by ClpP in response to ATP (2, 3). *In vitro*, in the presence of ClpP, ClpA promotes the degradation of RepA (the origin-binding protein of plasmid P1); but in the absence of ClpP, ClpA activates RepA for DNA binding by disassembling inactive dimers and releasing them in an active, monomeric form (4). Thus, it appears that the function of ClpA in proteolysis is to alter the conformational state of its substrates so that they become accessible to the protease. The Hsp104 protein of *Saccharomyces cerevisiae* is critical for survival after exposure to extreme temperatures (50 °C; Ref. 5) or high concentrations of ethanol (20%; Ref. 6). These stresses cause protein denaturation, and recent work indicates that Hsp104 promotes survival by facilitating the resolubilization of heat-damaged, aggregated proteins (7, 8).[1] Hsp104 also regulates the aggregation state of Sup35, the protein responsible for the prion-like inheritance of an extrachromosomal genetic element in yeast. Thus both ClpA and Hsp104 appear to utilize a similar mechanism for their widely divergent biological functions.

There are two classes of HSP100 proteins, and these are divided into eight subfamilies comprising more than 70 members identified to date (1). Hsp104 is a member of the B subfamily within class 1. The closest *E. coli* relative of Hsp104, ClpB, is a member of the same subfamily. It and many other members of this subfamily also function in thermotolerance (for review, see Ref. 1). The *E. coli* ClpA protein, too, is a class 1 protein but from a different subfamily. In biochemical terms, ClpA is currently the best characterized protein in this class.

All class 1 HSP100 proteins contain two predicted nucleotide-binding domains (NBD1 and NBD2)[2] separated by a middle region of variable size and flanked by amino- and carboxyl-terminal regions (1, 9). Both NBDs contain classical Walker-type consensus sequences for the P-loop (10) that resemble those of *N*-ethylmaleimide-sensitive fusion protein and the P-type transporter families. Both NBD1 and NBD2 are highly conserved in all class 1 HSP100 proteins but, except for the few residues that constitute the nucleotide-binding consensus, NBD1 and NBD2 exhibit little homology to each other. For example, although ClpA and Hsp104 are from different subfamilies, they share 51% identity in NBD1 and 42% identity in NBD2, but NBD1 and NBD2 of Hsp104 itself share only 22% identity. This high degree of sequence conservation in each NBD, throughout all four subclasses of class 1 HSP100 proteins, has led to the assumption that the functional relationships between the domains would also be conserved.

All class 1 HSP100 proteins tested assemble into homo-oligomers in the presence of adenine nucleotides (12–15) and hydrolyze ATP *in vitro* at similar rates (11, 12, and data herein). Both ClpA and Hsp104 assemble into ring-shaped hexamers, as determined by cross-linking, sizing chromatography, and electron microscopy (13–16). In every case tested, ATP is required for efficient functioning of the HSP100/Clp proteins *in vitro*. Moreover, point mutations in the NBDs, constructed to interfere with ATP binding or hydrolysis, eliminate biological

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[1] J. R. Glover and S. Lindquist, manuscript in preparation.

[2] The abbreviations used are: NBD, nucleotide-binding domain; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid.

function *in vivo* (17–19). Elucidating the contributions of the two NBDs to oligomerization and ATP hydrolysis is therefore an important first step for deciphering the molecular mechanism by which class 1 HSP100/Clp proteins alter the conformational state of their substrates.

For ClpA, two independent studies have addressed the contributions of the two NBDs to oligomerization and ATP hydrolysis. Both are in general agreement. One study concludes that NBD2 is not required for hexamer formation and that more than 50% of the basal ATPase activity is derived from NBD2 (18). The other study suggests that NBD1 is responsible for hexamer formation, whereas NBD2 is essential for ATP hydrolysis (19). For Hsp104, we previously reported that a point mutation in NBD1 had little effect on oligomerization *in vitro*, whereas an analogous mutation in NBD2 strongly inhibited oligomerization (15), a surprising contrast with results for ClpA. No characterization of the ATPase activity of wild-type Hsp104 and the effects of these NBD mutations on ATP hydrolysis has been presented. Here we present this characterization and examine the effects of environmental conditions relevant to the role of Hsp104 in stress tolerance on its ATPase activity. We also test the effects of additional NBD mutations on oligomerization and ATP hydrolysis. Mutations in the highly conserved residues of NBD2 exert stronger effects on oligomerization, whereas analogous mutations in NBD1 exert stronger effects on ATP hydrolysis, results that contrast with similar experiments in ClpA. The same relationship between NBD1 and NBD2 mutations was observed when ATP hydrolysis and oligomerization were examined in physiological ionic strength buffers and in the lower ionic strength buffer employed in ClpA studies. Thus, in agreement with work on ClpA, the two domains of Hsp104 have distinct biochemical characteristics. However, the ways in which similar mutations affect those characteristics are different in ClpA and Hsp104. The assumption that the sequence similarities in the NBD domains of ClpA and Hsp104 predicates an identical distribution of functions is reevaluated in the "Discussion."

EXPERIMENTAL PROCEDURES

Plasmid Construction—Point mutations that changed amino acids in the ATP-binding domains were produced by site-directed mutagenesis, using single-stranded plasmid DNA encoding the *HSP104* gene and the Muta-Gene M13 *in vitro* Mutagenesis Kit (Bio-Rad). The G217V and G619V coding sequences were inserted into the pJC45 vector (gift of J. Clos, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany), derived from the pJC20 vector (20) that contained a 10-histidine extension under control of the T7/lac promoter. To eliminate the possibility of extraneous mutations, K218T and K620T were produced in a modified version of the *HSP104* gene, $HSP104_R$, containing nucleotide substitutions that introduced restriction sites (*Bam*HI at −3, *Sac*II at 1125, *Sal*I at 1626, *Spe*I at 2128; the natural sites *Eag*I at 568 and *Sac*I at 2842 were also used so that unique sites occurred approximately every 500 base pairs) without changing the encoded amino acids or significantly altering codon usage. After mutagenesis, the segment containing the mutation was sequenced, excised, and inserted into an unmutagenized version of $HSP104_R$. The $K218T_R$ and $K620T_R$ coding sequences were inserted behind the 6-histidine extension of the pET28a vector (Novagen, Madison, WI) from which an encoded T7 epitope tag had been removed.

Protein Purification—Hsp104 was purified from yeast as described (15). For purification from *E. coli,* proteins were produced using the T7 expression system (21) pLysS strain (22) for K218T and K620T and using BL21[DE3](pAPlacIQ) (20) for the other mutants. Cells were grown to an A_{595} of <0.4 and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h. Induced cells were collected by centrifugation, sonicated in buffer A (20 mM Tris, pH 8.0, 400 mM NaCl, 10 mM imidazole), and bound to nickel-nitrilotriacetic acid-agarose (Qiagen, Chatsworth, CA). After washing in buffer A, proteins were eluted with the same buffer containing 220 mM imidazole, dialyzed against buffer B (20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 2 mM EDTA, 1.4 mM β-mercaptoethanol, 5% glycerol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride), and applied onto 5-ml DEAE columns (Amersham Pharmacia Biotech). Protein was eluted with a 50–300 mM KCl gradient in buffer B, dialyzed against buffer B containing 10% glycerol (storage buffer), and either frozen for long term storage or used immediately. WT, K218T, and K620T proteins were diluted to equal concentrations in the storage buffer to equalize potential inhibition by glycerol and in most assays were diluted into buffer at least 10-fold. In comparing the wider range of mutants, all proteins (including WT, K218T, and K620T) were first dialyzed against and concentrated in 20 mM HEPES, pH 7.5, 140 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol using Ultrafree-15 Centrifugal Filter Devices with a nominal molecular mass limit of 30,000 Da (Millipore, Bedford, MA). Concentrated proteins were diluted to equivalent concentrations with the same buffer.

Determination of Protein Concentration—Protein concentration was determined using the method of Bradford (23) with bovine serum albumin as a standard. Subsequently, an extinction coefficient was calculated for Hsp104 (A_{276}, ϵ = 31,900 M^{-1} cm^{-1}) that did not vary with native or denatured protein (24) and matched data from amino acid analysis but yielded lower values than the Bradford assay which overestimates Hsp104 protein concentration by a factor of ~2. Remaining frozen stocks of some proteins were analyzed using this extinction coefficient and the others extrapolated for the values given herein. Previously published work on Hsp104 utilized the Bradford assay standardized against bovine serum albumin, so values therein are overestimated.

ATPase Assays—General characterization of Hsp104 ATPase activity (Tables I–III and Fig. 1) was performed in reaction buffer 1 (40 mM Tris adjusted to pH 7.5 at 37 °C, 175 mM NaCl, 5 mM $MgCl_2$, 0.02% Triton X-100, and 5 mM ATP). For measurement of the ATPase activity at different pH values, 40 mM sodium acetate buffer was used for pH 4.5–6.0, 40 mM MOPS for pH 6.5–7.0, 40 mM Tris for pH 7.0–9.0, and 40 mM glycine-NaOH buffer for pH 9.5–10.5. Hsp104 was at a concentration of 0.01 mg/ml with a 7-min end point for all general characterization assays.

To relate oligomerization to ATP hydrolysis, assays comparing different mutant proteins and all K_m experiments were performed in the same buffer employed in oligomerization experiments, reaction buffer 2 (20 mM HEPES, pH 7.5, 140 mM KCl, 15 mM NaCl, 10 mM $MgCl_2$). K_m and V_{max} were identical whether assayed in reaction buffer 1 or 2, but HEPES buffer was employed because Tris buffer inhibits glutaraldehyde cross-linking. All reactions contained 5 mM ATP, pH 7.5, except for K_m experiments where ATP concentration was varied. The reaction buffer for K_m experiments included 5 mM $MgCl_2$, and ATP was resuspended with equimolar $MgCl_2$. All nucleotides were adjusted to pH 7.5 prior to ATPase assays. Experiments using low ionic strength buffers were performed in reaction buffer 3 (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.02% Triton X-100) or reaction buffer 2 minus salt.

All assays were performed at 37 °C in a 25-μl reaction volume in siliconized Eppendorf tubes so that Triton X-100 (used in reaction buffer 1 to reduce loss of proteins on the walls of reaction tubes) could be eliminated; this detergent slightly stimulates the ATPase activity of Hsp104. Reactions were terminated and released P_i quantified by the addition of 800 μl of Malachite Green Reagent (0.034% Malachite Green, catalog number M9636, Sigma; 1.05% ammonium molybdate; 1 M HCl, filtered to remove insoluble material; Ref. 25). After 1 min at room temperature, color development was stopped by addition of 100 μl of 34% citric acid. 200 μl of the sample was removed to 96-well assay plates and A_{650} determined with a Molecular Devices (Palo Alto, CA) vmax kinetic microplate reader with SoftMax™ software. Values were calibrated against KH_2PO_4 standards and corrected for phosphate released in the absence of Hsp104. For each point at least 3 and typically 6 independent assays were used to calculate the mean ± S.D. Curves were generated using least squares fitting to the Michaelis-Menten equation with the Kaleidagraph™ graphics program (Synergy Software, Reading, PA). K_m and V_{max} were calculated using the Kaleidagraph program, and similar values were also obtained using Lineweaver-Burk and Eadie-Hofstee plots.

Cross-linking Assays—WT and mutant proteins dialyzed against reaction buffer 2 with 2 mM dithiothreitol were cleared of aggregated protein by centrifugation and adjusted to 0.01 mg/ml in reaction buffer 2 containing dithiothreitol (2 mM) with or without ATP (5 mM). 200-μl reactions were incubated at 37 °C for 10 min, glutaraldehyde added (final concentration 0.01%), and cross-linking terminated at 0 or 12 min with 1 M glycine as described (15). Reaction buffer 2 lacking KCl and NaCl was employed for cross-linking reactions at low ionic strengths.

RESULTS

Characterization of Wild-type Hsp104 ATPase Activity—First, we characterized the ATPase activity of WT Hsp104

TABLE I
The ability of other cations to replace Mg^{2+} in supporting ATP hydrolysis by Hsp104

All ATPase assays for general characterization experiments were performed in reaction buffer 1 at 37 °C for 7 min with 5 mM ATP and 0.01 mg/ml Hsp104. Here, the Mg^{2+} in the reaction buffer was replaced with the indicated ion at 5 mM. Values for the *E. coli* HSP100 proteins ClpA and ClpB are from previous reports.

Ion	Hsp104	Hsp104[a]	ClpA %[b]	ClpB %[c]
	nmol P_i released	%		
	0.00 ± 0.05	0		
Mg^{2+}	0.97 ± 0.12	100	100	100
Mn^{2+}	1.69 ± 0.25	175	17	100
Ca^{2+}	0.01 ± 0.01	0	50	25
Cu^{2+}	0.04 ± 0.09	0	0	0
Zn^{2+}	0.16 ± 0.05	15	0	0
Fe^{2+}	0.00 ± 0.05	0	0	0
Co^{2+}	4.13 ± 0.68	425	0	0
Cd^{2+}	0.02 ± 0.02	0		
Ni^{2+}	1.27 ± 0.34	130		

[a] % of activity with Mg^{2+}.
[b] Hwang *et al.* (3).
[c] Woo *et al.* (12).

using a colorimetric assay to measure phosphate released. ATP hydrolysis required divalent cations. Hydrolysis was supported by Mg^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} but not by Ca^{2+}, which supports hydrolysis by ClpA and ClpB (3, 12) (Table I). Surprisingly, hydrolysis in the presence of 5 mM $CoCl_2$ was 4-fold higher than with 5 mM $MgCl_2$. Although the closest *E. coli* homolog of Hsp104, ClpB, is reported to hydrolyze other nucleotides (12) (Table II), Hsp104 did not hydrolyze other nucleotides at a significant rate (Table II). GTP, CTP, and UTP moderately inhibited the ATPase activity of Hsp104, whereas AMP inhibited it only slightly. ADP, however, inhibited the ATPase activity of Hsp104 strongly (Table III).

Because Hsp104 promotes survival after exposure to extreme stresses (high temperatures or high concentrations of ethanol) and because the functions of both nucleotide-binding sites are required for stress tolerance (17), we examined the effects of heat and ethanol on the ATPase activity of Hsp104 *in vitro* (Fig. 1). Initially, as temperature increased, Hsp104 ATPase activity increased. However, this increase was no greater than expected for a typical enzyme system; for every 10 °C increase in temperature, activity roughly doubled. At temperatures above 45 °C, activity dropped precipitously (Fig. 1*A*). Ethanol inhibited ATPase activity at all concentrations tested, eliminating it at a concentration of 20% (Fig. 1*B*).

Because the intracellular pH of yeast cells drops during heat stress (26), we also tested the ATPase activity of Hsp104 at different pH values (Fig. 1*C*). Hsp104 hydrolyzed ATP between pH 5 and 10. Within this range, the nadir for ATPase activity occurred at physiological pH (7.5). Maximal activity occurred at pH 6.5 (200% of the activity at pH 7.5); a second optima was observed at pH 9.0 (150% of the activity at pH 7.5).

Hsp104 exhibited Michaelis-Menten kinetics over an ATP concentration range of 0.5 to 30 mM. (Deviations from Michaelis-Menten kinetics occurred at nucleotide concentrations lower than 0.5 mM.)[3] Between 0.5 and 30 mM ATP (pH 7.5, 37 °C, physiological salt), Hsp104 hydrolyzed ATP with a K_m of ~5 mM and a V_{max} of ~2 nmol of P_i min^{-1} μg^{-1} (Fig. 2). This corresponds to a turnover rate of ~20 ATP molecules per hexamer of Hsp104 per s. Given that the two nucleotide-binding domains of Hsp104 have very different sequences, if hydrolysis at both sites makes a substantial contribution to phosphate released, a plot of *V versus* ATP concentration might be expected to yield a biphasic (sigmoidal) curve. This was not observed. It is possible that both domains contribute to ATP hydrolysis over this range of ATP concentrations but that the two NBDs, although very different in primary amino acid sequence, have very similar affinities for ATP. Alternatively, one of the two domains may contribute most of the ATP hydrolysis activity of the protein.

TABLE II
The ability of Hsp104 to hydrolyze various nucleotides

ATP in reaction buffer 1 was replaced with the different nucleotides, all at a concentration of 5 mM. Reactions were performed as in Table I. Values for ClpA and ClpB are from previous reports. Only ATP was hydrolyzed by Hsp104 at a significant level.

Nucleotide	Hsp104	Hsp104	ClpA	ClpB
	nmol P_i released	%[a]	%[b]	%[c]
ATP	2.08 ± 0.65	100	100	100
ADP	0.04 ± 0.07	0	0	0
AMP	0.01 ± 0.02	0	0	0
GTP	0.03 ± 0.05	0	0	23
CTP	0.06 ± 0.04	3	0	15
UTP	0.01 ± 0.02	0	0	16

[a] % of value obtained with ATP.
[b] Hwang *et al.* (3).
[c] Woo *et al.* (12).

TABLE III
The ability of other nucleotides to inhibit ATP hydrolysis by Hsp104

Hydrolysis of ATP (5 mM) was examined in the presence of other nucleotides (each also at 5 mM). ADP inhibited ATP hydrolysis by Hsp104 7-fold.

Nucleotide	nmol P_i	%[a]
	2.08 ± 0.65	100
ADP	0.31 ± 0.06	15
AMP	1.90 ± 0.44	90
GTP	0.71 ± 0.32	35
CTP	0.87 ± 0.34	40
UTP	0.63 ± 0.29	30

[a] % of value obtained with ATP alone.

ATP Hydrolysis by hsp104 Point Mutants—To investigate the relative contributions of each nucleotide-binding site to ATP hydrolysis, we examined the effects of point mutations in nucleotide-binding consensus residues (Fig. 3*A*). One matching set of mutants, K218T and K620T, carried threonine substitutions in the conserved lysine that interacts directly with the β- and γ- phosphates of bound nucleotide and is important for the structure of the P-loop in other nucleotide-binding proteins with Walker consensus motifs (27). Separately, matching glycine to valine substitutions (G217V,G619V) were generated in the conserved glycines immediately preceding these lysines. All of these residues are absolutely conserved in both the Walker A P-loop consensus ((G/A)X_4GK(T/S)) (10, 27) and in the more specific HSP100 family consensus (GX_2GXGKT) of both NBDs. When proteins carrying the lysine to threonine substitutions were expressed in yeast cells, they were no more susceptible to proteolysis than WT Hsp104, suggesting the mutations caused no global perturbation in structure (15). Furthermore, the circular dichroism spectra of both the K218T and the K620T mutants were very similar to WT protein, also indicating no gross loss of structure in the mutants (data not shown).

To simplify purification procedures, *HSP104* coding sequences were cloned into expression vectors encoding amino-terminal histidine extensions, and the proteins were purified from *E. coli* by chromatography on nickel resin, followed by chromatography on DEAE. Histidine-tagged Hsp104 provided wild-type levels of thermotolerance when expressed in a yeast *hsp104* deletion strain.[4] Moreover, the ATPase activity and oligomerization properties of the WT protein purified from

[3] E. C. Schirmer and S. Lindquist, unpublished results.
[4] D. A. Parsell and S. Lindquist, unpublished observations.

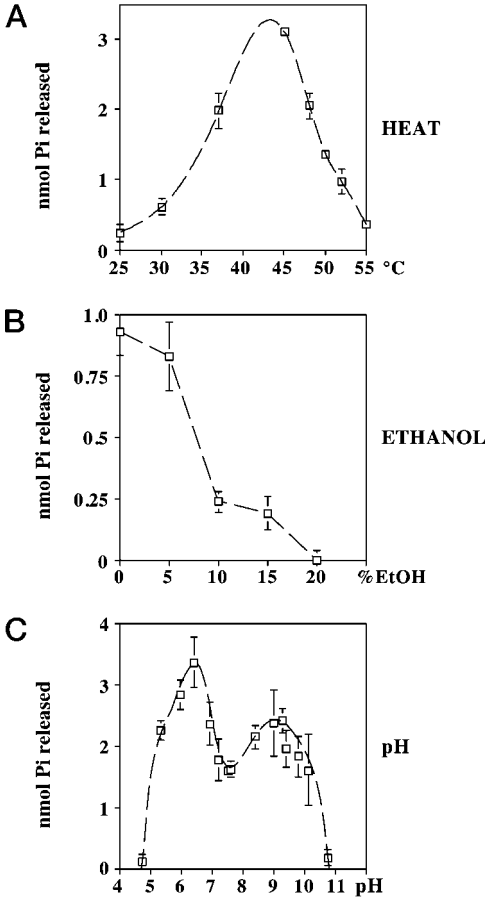

FIG. 1. **Effects of stress conditions on ATP hydrolysis by WT Hsp104.** Assays were performed as described in Table I, and activity is plotted as nanomoles of phosphate released by Hsp104 (0.25 μg per reaction; 0.01 mg/ml) after 7 min (within the linear range of the assay). *A*, effects of temperature. Hydrolysis decreases above 45 °C. *B*, effects of ethanol. Hydrolysis declines sharply above 5%. *C*, effects of pH. Hydrolysis is reduced at the normal cellular pH (7.2–7.5).

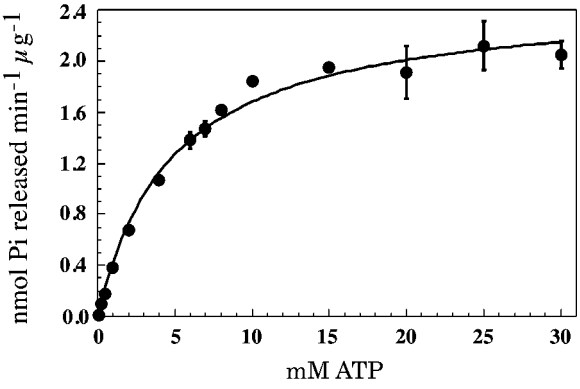

FIG. 2. **Kinetic analysis of ATP hydrolysis by WT Hsp104.** All kinetic assays were performed in the same buffer as cross-linking studies (reaction buffer 2) at 37 °C. The rate of hydrolysis was calculated from the nanomoles of phosphate released by 0.5 μg of Hsp104 (0.02 mg/ml) during the first 5 min of incubation with ATP at various concentrations. The K_m of ~5 mM and the V_{max} of ~2 nmol min^{-1} μg^{-1} were determined by least squares fitting to the Michaelis-Menten equation using the Kaleidagraph program (Synergy Software). Similar values were obtained using Lineweaver-Burk and Eadie-Hofstee plots.

yeast (15) were very similar to those of the protein purified from *E. coli*, whether the histidine residues remained attached or were cleaved from the protein with thrombin (data not shown). The purity of mutant proteins employed in this analysis is shown in Fig. 3*B*.

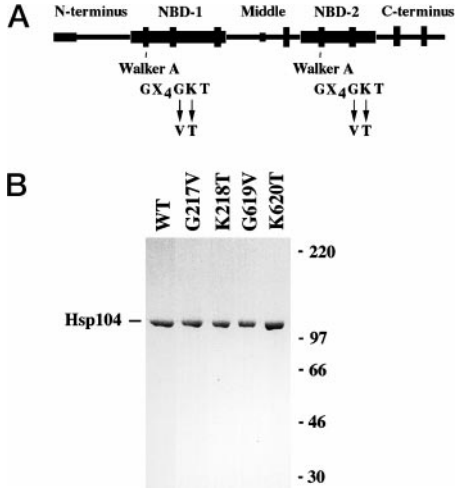

FIG. 3. **Positions of mutations and purity of proteins.** *A*, general structural features of Hsp104 and the location of mutations employed in this study. Sequence regions that are highly conserved in the HSP100 family, including the Walker A and B motifs, are indicated by *thick lines*. The P-loop (Walker A) consensus sequences are given, and positions of mutations therein are indicated by *arrows* pointing to the substituted amino acid. *B*, the purity of proteins expressed in *E. coli*. Hsp104 proteins carrying a histidine extension were purified as described under "Experimental Procedures," resolved on 7.5% SDS-polyacrylamide gels and stained with Coomassie Blue dye. Hsp104 proteins appeared to be >98% pure. The migration of molecular weight standards is indicated on the *right*.

Proteins carrying mutations in NBD1 (G217V or K218T) did not hydrolyze ATP at measurable rates (Fig. 4, *inset*). In contrast, proteins carrying equivalent NBD2 substitutions (G619V or K620T) did hydrolyze ATP (Fig. 4, *inset*), albeit at a reduced rate, ~5–10% that of the wild-type protein (Fig. 4). As discussed below, low ionic strength buffers increased hydrolysis by the NBD2 mutants. Furthermore, the NBD2 mutants exhibited an oligomerization defect, and when that defect was overcome by high protein concentrations, ATP hydrolysis increased to near wild-type levels.[5] In contrast, ATP hydrolysis by the NBD1 mutants remained negligible at high protein concentrations. Thus the NBD1 mutation inhibited ATPase activity more completely than the NBD2 mutation.

Oligomerization of hsp104 Point Mutants—We examined the oligomerization properties of one of these mutant pairs previously, finding that a lysine to threonine substitution in NBD2 (K620T) impaired the protein assembly into hexamers in response to ATP, whereas the analogous substitution in NBD1 (K218T) had little effect (15). Surprisingly, an analogous lysine to threonine substitution in the NBD1 of ClpA impaired the assembly of the proteins into hexamers in response to ATP, whereas the analogous substitution in NBD2 had little effect (19). Here, to directly relate oligomerization and ATP hydrolysis, we compared the effects of the Lys → Thr substitutions, as well as the new Gly → Val substitutions, on oligomerization using the same buffer employed in ATPase assays. This buffer mimics physiological ionic strength (140 mM KCl and 15 mM NaCl). The G619V substitution, like the K620T substitution, impaired oligomerization of Hsp104; the G217V substitution, like the K218T substitution, did not (Fig. 5). Thus, substitutions in highly conserved nucleotide-binding consensus residues of the NBD2 of Hsp104 affect oligomer assembly more than the same substitutions in NBD1.

Effects of Salt Concentration on the ATPase Activity of Hsp104—We characterized the ATPase activity of Hsp104 us-

[5] E. C. Schirmer, C. Queitsch, A. S. Kowal, and S. Lindquist, manuscript in preparation.

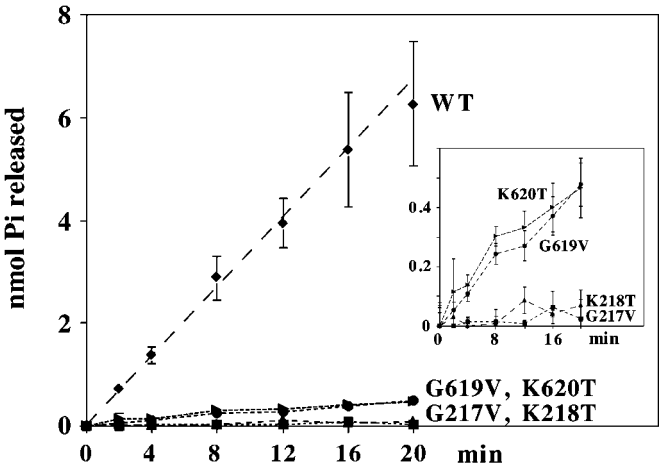

FIG. 4. **ATPase activity of mutant Hsp104 proteins.** ATPase assays were performed as in Fig. 2, under the conditions employed in cross-linking studies (see Fig. 5). Reactions containing 0.5 μg of Hsp104 (0.02 mg/ml) were incubated at 37 °C for the times indicated, and nanomoles of phosphate released are shown. ATP hydrolysis by K218T and by G217V was negligible at all time points (nearly all *error bars* overlapped with the zero value, see *inset*), whereas the K620T and G619V mutants clearly exhibited significantly higher levels of ATP hydrolysis (see *inset*). Nonetheless, these values were reduced, reaching only 5–10% of wild-type levels. Under other conditions (Fig. 7) higher levels of hydrolysis were observed for NBD2 mutants, but hydrolysis by the NBD1 mutants remained negligible. The effect was observed with multiple preparations of the hsp104 mutants and also with K218T and K620T proteins purified from yeast without a histidine extension. The G619V and K620T mutants had strikingly similar activities, so that it is difficult to distinguish them in the larger graph.

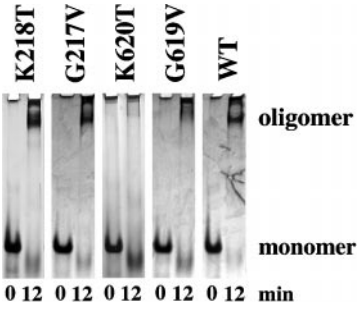

FIG. 5. **Effects of mutations on the oligomerization of Hsp104 protein.** Mutant proteins (at 0.01 mg/ml) were incubated for 0 or 12 min at 37 °C with 5 mM ATP, cross-linked with glutaraldehyde, separated on 3.5% SDS-polyacrylamide gels, and stained with silver. The K218T and G217V mutants oligomerized indistinguishably from WT Hsp104, but the K620T and G619V mutants were deficient in oligomerization.

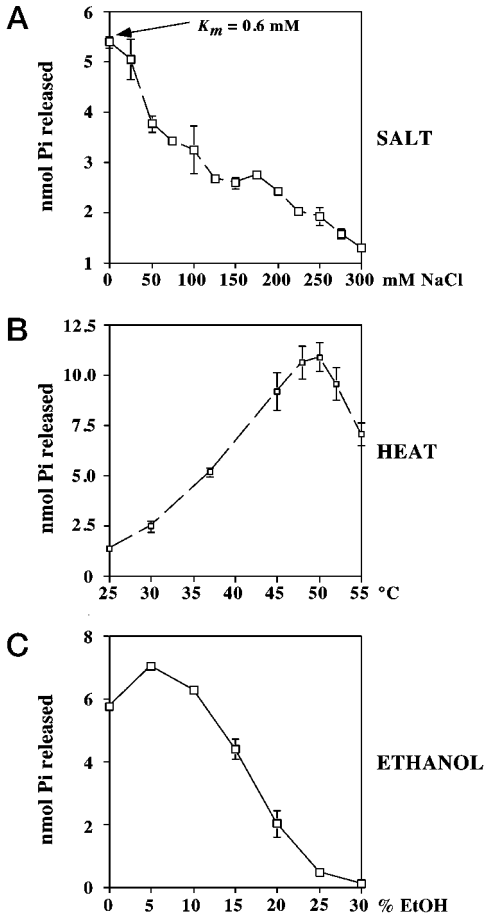

FIG. 6. **Effects of salt on Hsp104 ATPase activity.** *A*, effect of increasing salt concentration on the basal rate of ATP hydrolysis. As NaCl concentration increased, ATP hydrolysis was inhibited. The K_m measured in buffer containing no NaCl is indicated by the *arrow*. The K_m of Hsp104 (0.02 mg/ml) measured at 140 mM KCl and 15 mM NaCl (155 mM salt) was ~5 mM. *B*, effects of temperature. ATP hydrolysis at different temperatures was assayed as in Fig. 1 but in the absence of added salt. Under these conditions the ATPase activity was sustained during the stress, reaching a maximum at 50 °C in contrast with measurements in physiological salt where hydrolysis had dropped to <½ maximal activity at 50 °C. *C*, effects of ethanol. The ability of Hsp104 to hydrolyze ATP in the presence of ethanol was also increased in the low ionic strength buffer (compare with Fig. 1*B*).

ing buffers that mimic physiological conditions; however, Maurizi and colleagues (14) reported that the ATPase activity of ClpA was inhibited by high salt concentrations, and most published work on other HSP100 proteins has used low ionic strength buffers. To compare the ATPase activity of Hsp104 with that of other HSP100 proteins and to determine if the different effects of the ClpA and the Hsp104 mutations are due to the use of different buffer conditions, we also examined ATPase activity and oligomerization properties of Hsp104 in low ionic strength buffers.

The salt content of buffers strongly influenced ATPase activity. As the ionic strength of the buffer increased, ATPase activity decreased (Fig. 6*A*). In separate experiments the K_m of Hsp104 was tested in buffer lacking added NaCl and KCl. The K_m measured by ATP hydrolysis dropped to 0.6 mM in this low salt buffer, compared with a K_m of ~5 mM in the physiological salt buffer (Fig. 7). The V_{max} was unaffected.

The ability of Hsp104 to hydrolyze ATP was also more stable to stressful conditions in low salt buffer. Maximal ATP hydrolysis occurred at 50 °C rather than 40 °C (Fig. 6*B*, compare with Fig. 1*A*), and ATP hydrolysis was actually stimulated with 10% ethanol in contrast with the >50% inhibition observed in the physiological buffer (Fig. 6*C*, compare with Fig. 1*B*). Varying the ionic strength of buffers did not, however, change the effects of pH on the ATPase activity of Hsp104 (data not shown).

The changes in the ATPase activity of Hsp104 in low salt buffers may be related to structural changes in the protein. At the protein concentrations employed here, WT Hsp104 does not assemble into hexamers in physiological buffer unless nucleotides are present. In low salt buffer Hsp104 assembles into hexamers in the absence of nucleotides (as determined by glutaraldehyde cross-linking, data not shown). Since WT Hsp104 is oligomeric at all nucleotide concentrations over which K_m values were measured, the different K_m values in low and physiological salt buffers cannot be due to oligomerization *per se*. One explanation may be that ionic interactions involved in nucleotide binding weaken as the salt content of buffers increases, thus increasing K_m values. However, the low salt buffer may also induce a conformational change that both promotes oligomerization and lowers the K_m in the oligomer.

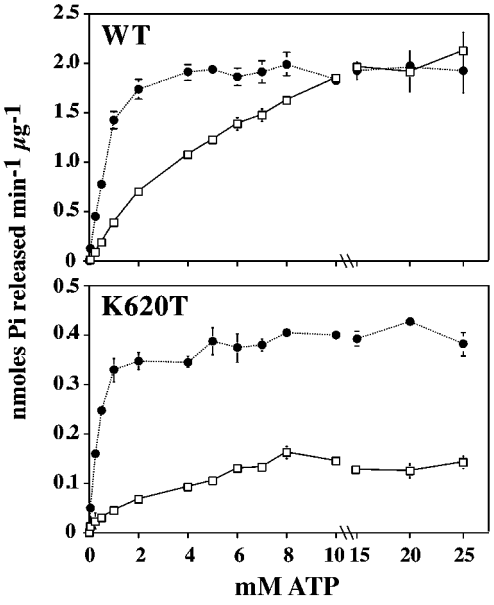

FIG. 7. **Effects of salt on WT and K620T mutant proteins.** WT or K620T proteins, both at 0.02 mg/ml, were incubated with increasing concentrations of ATP for 16 min at 37 °C. Nanomoles of phosphate released were determined, corrected for background, and adjusted to min^{-1} μg^{-1} for the graphs shown. The K_m of the WT protein decreased nearly 10-fold in the absence of salt (*filled circles*), but the V_{max} remained the same. The K_m of the K620T mutant was affected by salt (*open squares*) in the same manner as WT protein, but the V_{max} of the mutant increased 3–4-fold in the absence of salt. Thus, the defect in ATP hydrolysis in the NBD2 mutant is diminished under conditions of low ionic strength.

The effects of the NBD mutations were also assessed in the low salt buffer. The K218T mutant still displayed no measurable ATP hydrolysis. However, the ATP hydrolysis defect of the K620T protein was diminished; ATPase activity increased from 5 to 10% of WT levels, observed at physiological ionic strength, to 20–30% of WT levels (Fig. 7). The K_m of the K620T mutant was similar to that of the wild-type protein in physiological salt and decreased to a similar extent in low salt. However, whereas the V_{max} of the WT did not increase in low salt, the V_{max} of the K620T mutant increased 3–4-fold (Fig. 7). Under these conditions the distinction between the effects of the NBD1 mutation and the NBD2 mutation on ATP hydrolysis is even greater than it was in the physiological ionic strength buffer. Thus, the differences in the effects of our mutations and those reported for similar ClpA mutations are not rectified by changing the ionic strength of the buffer.

DISCUSSION

We have provided the first characterization of the ATPase activity of WT Hsp104 from *S. cerevisiae* and the effects of environmental conditions related to its biological function on that activity. We have also shown that mutations in conserved residues of the first nucleotide-binding domain of Hsp104 (NBD1) severely reduce the ATPase activity of Hsp104 but do not inhibit oligomerization, whereas analogous mutations in the second nucleotide-binding domain (NBD2) have a modest effect on ATP hydrolysis but inhibit Hsp104 oligomerization. Although the oligomerization properties and ATPase activity of WT Hsp104 are dramatically affected by differences in the ionic strength of buffers, the distinct effects of NBD1 and NBD2 mutations are retained.

Because the nucleotide-binding domains of Hsp104 are essential for its function in stress tolerance (17), we also examined the effects of parameters that vary with stress on ATPase activity of Hsp104. In physiological buffers, the ATPase activity of Hsp104 peaks at about 45 °C and is greatly reduced at 55 °C. It is inhibited by ethanol, very severely at 20%. Strikingly, it is in cells exposed to such conditions that Hsp104 is most critical for survival. The expression of Hsp104 increases survival in cells exposed to 44 °C by 10-fold but increases survival in cells exposed to 55 °C by 1000-fold (28). A similar relationship holds for ethanol (6). This result provides biochemical support for an earlier suggestion, based on very different types of evidence, that the key function of Hsp104 is to repair damage after stress, rather than to prevent damage during stress (7, 8). However, we also find that in low salt buffers, Hsp104 retains ATPase activity at higher temperatures and higher concentrations of ethanol. Thus, it remains possible that as yet undiscovered *in vivo* factors mimic this effect and allow Hsp104 to function even during severe stress.

The effects of pH, ATP concentrations, and ATP/ADP ratios may also be relevant to the biological function of Hsp104. ATP hydrolyzing activity is high at pH 6.5 and low at pH 7.5. ^{31}P NMR measurements of chemical shifts in inorganic and sugar phosphates indicate that the cytoplasmic pH of yeast cells decreases from 7.5 at 23 °C to 7.0 at 37 °C and subsequently to 6.2 at 45 °C (26). Such changes in pH may increase the activity of Hsp104 during or immediately after stress and inhibit it again when normal physiology is restored. The K_m for ATP hydrolysis by Hsp104 is ~5 mM, close to the normal physiological concentration of ATP (~4 mM; Refs. 29 and 30). The nucleotide hydrolyzing activity of Hsp104 is highly specific for ATP and is strongly inhibited by ADP. Thus, stress-induced fluctuations in ATP concentrations and ATP/ADP ratios may also regulate the activity of Hsp104 *in vivo*.

The K_m of Hsp104 at physiological ionic strength is 20-fold higher than the K_m reported for ClpA (Table IV). However, the buffers employed for K_m measurements of ClpA did not contain NaCl or KCl (3). When the K_m of Hsp104 was measured in a low ionic strength buffer, it was reduced 10-fold. Thus, under similar conditions, these different HSP100 proteins have very similar K_m values. The V_{max} of Hsp104 was not much affected by ionic strength and is similar to values reported for ClpA. The similarities in the ATPase activities of these two proteins underscore other, previously noted similarities as follows: (*a*) both form hexameric complexes in the presence of ATP (15, 16, 18,

TABLE IV
Comparison of our data on Hsp104 with published K_m and V_{max} values for other members of the HSP100 family

Although the K_m of Hsp104 for ATP hydrolysis in physiological buffer was high compared to values given or predicted for other HSP100s, when tested under similar buffer conditions similar values were obtained. Values were taken from the listed publications or inferred from the data presented when values were not given. Although no kinetic analysis has been reported for the ClpC protein of *B. subtilis*, a rate of 0.4 nmol min^{-1} μg^{-1} is inferred from the data presented by Turgay *et al.* (31).

Hsp	Organism	K_m	V_{max}
		mM	*nmol* min^{-1} μg^{-1}
HSP100/Clp class 1			
Hsp104, phys salt	*S. cerevisiae*	5	2
Hsp104, low salt	*S. cerevisiae*	0.6	2
ClpA[a]	*E. coli*	0.2	0.7–0.9
ClpB[b]	*E. coli*	1.1	0.8[c]
HSP100/Clp class 2			
AmiB[d]	*P. aeruginosa*	0.174	2.4
ClpX[e]	*E. coli*	0.5	0.5[f]

[a] Hwang *et al.* (3) and Maurizi *et al.* (14).
[b] Woo *et al.* (12).
[c] Inferred from Woo *et al.* (12).
[d] Wilson *et al.* (32).
[e] Wawrzynow *et al.* (33).
[f] Inferred from Wawrzynow *et al.* (33).

19); (*b*) both promote the disassembly of protein aggregates or oligomers (4, 7); (*c*) both contain two nucleotide-binding domains that have little sequence homology with each other but are highly related to the corresponding domains of the other protein (1, 9).

Given the similarities between ClpA and Hsp104, it is surprising that analogous mutations in their nucleotide-binding domains have such different effects on their most basic biochemical characteristics, oligomerization and ATP hydrolysis. In Hsp104, a lysine to threonine mutation in the P-loop of NBD1 (K218T) inhibits ATP hydrolysis more severely than the equivalent mutation in NBD2 (K620T), whereas the mutation in NBD2 inhibits oligomerization more severely than the equivalent mutation in NBD1. In ClpA the opposite relationship was reported; a lysine to threonine P-loop substitution in NBD1 most strongly affects oligomerization, whereas the equivalent mutation in NBD2 more strongly affects ATP hydrolysis (19).

We investigated this apparent contradiction in three ways. 1) Because site-directed mutagenesis might have introduced extraneous mutations, we recreated the K218T and K620T mutations in a manner that ensured that no extraneous mutations were present (see "Experimental Procedures," $HSP104_R$). It seems unlikely that extraneous mutations in the ClpA proteins caused this discrepancy, as multiple mutants were tested in two different laboratories (18, 19). 2) We tested additional mutations in the NBDs, specifically, equivalent glycine to valine substitutions in the P-loop consensus sequences of each NBD. These mutants behaved similarly to our original lysine to threonine substitutions. 3) We used both low salt buffers and physiological ionic strength buffers for ATPase and oligomerization assays. (ClpA oligomerization assays had been performed in buffers containing 100–300 mM NaCl or KCl, whereas ATPase assays were performed in the absence of added salt (18, 19).) Although the ionic strength of the buffer affected both the oligomerization properties and ATPase activity of Hsp104, the NBD2 mutations still exerted stronger effects on the oligomerization of Hsp104, and the NBD1 mutations still exerted stronger effects on ATP hydrolysis.

One explanation for the different effects of equivalent mutations in ClpA and Hsp104 is that the two domains have independent functions, one responsible for ATP hydrolysis and the other for oligomerization, and that these functions have switched in the two proteins during the course of evolution. This switch might be due to the influence of specific amino acid substitutions in the nucleotide-binding domains or to the influence of flanking domains, which are highly divergent in different HSP100 subtypes. A precedent exists for similar nucleotide-binding domains evolving such diverse functions in *N*-ethylmaleimide-sensitive fusion protein, a protein that regulates vesicle fusion. *N*-ethylmaleimide-sensitive fusion protein has two NBDs that are similar to each other in sequence, yet nucleotide binding at one site is required for oligomerization, whereas ATPase activity at the other site is required for function. Furthermore, the order of the domains can be switched without altering their functions (34).

An alternative explanation is that both domains contribute to ATP hydrolysis and oligomerization in a complex, interdependent manner and that other differences between the two proteins idiosyncratically cause analogous mutations to perturb one of these functions more than the other. At least three lines of evidence support this notion. First, in both ClpA (18) and Hsp104,[5] oligomerization increases ATPase activity. Second, class 2 HSP100 proteins have only one NBD, which more closely resembles the NBD2 of class 1 proteins, yet mutational analysis indicates that it functions both in oligomerization and ATP hydrolysis (35). Third, oligomerization occurs at very low nucleotide concentrations (μM ATP or ADP)[6] such that *in vivo* nucleotide concentrations (mM ATP or ADP) should always be sufficient to maintain WT protein in an oligomerization-competent state. Thus, it seems likely that nucleotide binding (and perhaps hydrolysis) at the second site serves some other purpose beyond simply tethering the subunits together.

Understanding the reaction cycle utilized by Hsp104 will require a detailed understanding of the interplay between these sites. The multimeric nature of the protein, the possibility that both NBDs may contribute at different levels to ATP hydrolysis and oligomerization, the effects of environmental conditions on its behavior, and the as yet poorly explored effects of partner proteins and substrates make this a challenging problem. Results presented here provide the first step.

Acknowledgments—We thank Edward Taylor, John Westley, Anil Cashikar, and John Glover for helpful discussions. We thank Joachim Clos for the kind gift of pJC45 vector and Olivier Fayet for strain BL21[DE3](pAPlacIQ). We thank Lisa Rosman, Laura Loftus, and Danielle Ware for technical assistance.

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Additions and Corrections

Vol. 272 (1997) 32534–32538

ϵ-Sarcoglycan, a broadly expressed homologue of the gene mutated in limb-girdle muscular dystrophy 2D.

Audrey J. Ettinger, Guoping Feng, and Joshua R. Sanes

Page 32535, Fig. 1*B*: 24 nucleotides were inadvertently omitted. Inclusion of the nucleotides yields a cDNA of 1548 nucleotides total. The additional nucleotides are in **bold type** (GenBank™/EBI accession number AF031919).

FIG. 1.

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The ATPase activity of Hsp104, effects of environmental conditions and mutations.

Eric C. Schirmer, Christine Queitsch, Anthony S. Kowal, Dawn A. Parsell, and Susan Lindquist

Page 15552: A reference was omitted. The last three references, in correct numerical order, are:

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