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Functional Analysis of Conserved *Cis*- and *Trans*-elements in the Hsp104 Protein Disaggregating Machine

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Abstract

Hsp104 is a double ring-forming AAA+ ATPase, which harnesses the energy of ATP binding and hydrolysis to rescue proteins from a previously aggregated state. Like other AAA+ machines, Hsp104 features conserved *cis*- and *trans*-acting elements, which are hallmarks of AAA+ members and are essential to Hsp104 function. Despite these similarities, it was recently proposed that Hsp104 is an atypical AAA+ ATPase, which markedly differs in 3D structure from other AAA+ machines. Consequently, it was proposed that arginines found in the non-conserved M-domain, but not the predicted Arg-fingers, serve the role of the critical *trans*-acting element in Hsp104.

While the structural discrepancy has been resolved, the role of the Arg-finger residues in Hsp104 remains controversial. Here, we exploited the ability of Hsp104 variants featuring mutations in one ring to retain ATPase and chaperone activities, to elucidate the functional role of the predicted Arg-finger residues. We found that the evolutionarily conserved Arg-fingers are absolutely essential for ATP hydrolysis but are dispensable for hexamer assembly in Hsp104. On the other hand, M-domain arginines are not strictly required for ATP hydrolysis and affect the ATPase and chaperone activities in a complex manner. Our results confirm that Hsp104 is not an atypical AAA+ ATPase, and uses conserved structural elements common to diverse AAA+ machines to drive the mechanical unfolding of aggregated proteins.

Keywords

AAA+ ATPase; Chaperone; Disaggregase; Hsp104; Arginine-finger

Introduction

The ability of bacteria, fungi, and plantae to recover functional protein from aggregates is essential for their cell survival. The Hsp104 AAA+ ATPase is the principal protein disaggregase in *Saccharomyces cerevisiae*. Hsp104 and its bacterial homolog ClpB recognize aggregated proteins and recover functional protein in cooperation with the cognate Hsp70 system (Hsp70/40) (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Motohashi

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et al., 1999; Zolkiewski, 1999). As a consequence, Hsp104 is essential for thermotolerance development in yeast (Sanchez and Lindquist, 1990), and crucial to the yeast stress response (Parsell et al., 1994; Sanchez et al., 1992).

In addition to its role in protein disaggregation, Hsp104 is also essential for the inheritance and maintenance of yeast prions (Chernoff et al., 1995; Moriyama et al., 2000). It has been proposed that the ability of Hsp104 to remodel amorphous aggregates and prion fibrils relies on a common mechanism (Tessarz et al., 2008; Tipton et al., 2008). Interestingly, despite a similar 3D structure (Lee et al., 2010), the ability to remodel prions is not shared with bacterial ClpB (Inoue et al., 2004; Shorter and Lindquist, 2004), suggesting functional and perhaps mechanistic differences between yeast Hsp104 and bacterial ClpB.

At the molecular level, Hsp104 contains an N-terminal domain followed by two tandem AAA+ domains, and an acidic C-terminal domain unique to eukaryotic Hsp104 members (Fig. S1). The C-terminal tail of Hsp104 features an EIDDDL motif reminiscent of the EEVD motif present in Hsp70 and Hsp90, and is required for the interaction of Hsp104 with Hsp90 co-chaperones (Abbas-Terki et al., 2001; Moosavi et al., 2010) and with components of the GET pathway that mediate the sorting of tail-anchored proteins to the ER membrane (Wang et al., 2010). The first ATP-binding cassette (AAA-1) features the middle (M)-domain, a hallmark of Hsp104 and ClpB. The X-ray structure of ClpB showed that the M-domain consists of an 85Å-long coiled-coil inserted within the small α -helical domain of AAA-1, and can be further subdivided into two smaller coiled-coils, termed motif 1 and motif 2 (Lee et al., 2003). While most other Hsp100 proteins lack an M-domain, ClpC features a shorter M-domain (Fig. 1A and S1). The structure of the ClpC M-domain is reminiscent of motif 1 of ClpB/Hsp104, and is essential for interaction with the ClpC-specific adaptor protein MecA (Wang et al., 2011). In Hsp104, the M-domain is essential for protein disaggregation (Sielaff and Tsai, 2010), mediates the cooperative interaction with the cognate Hsp70 system (Miot et al., 2011; Sielaff and Tsai, 2010), and is required for inter-domain communication between the AAA-1 and AAA-2 domains (Cashikar et al., 2002). However, the Hsp104 M-domain is not essential for the Hsp104 ATPase activity (Sielaff and Tsai, 2010), consistent with the notion that other Hsp100 proteins which lack an M-domain, such as ClpA, are functional ATPases (Kress et al., 2009).

The fitted cryoEM structures of full-length Hsp104 and an N-terminal domain-truncated Hsp104 variant showed that the 3D structure of yeast Hsp104 and bacterial ClpB is conserved (Lee et al., 2007; Lee et al., 2010; Lee et al., 2003). As seen in other AAA+ machines, the ATP-binding site is formed at the interface between adjacent subunits in the homo-hexamer with the M-domains projecting outwards from the main body (Fig. 1B and C) (Lee et al., 2010). This subunit arrangement creates a narrow, axial channel that traverses the Hsp104 hexamer through which unfolded polypeptides are threaded (Lum et al., 2004; Tessarz et al., 2008). In addition, from the cryoEM-fitted structure (Lee et al., 2010), the location of conserved structural elements, such as the Walker A and B motifs, the sensor 1 and 2 motifs, and the Arg-finger residues, which are a hallmark of AAA+ machines, can be readily identified (Fig. 1B and C insets, and data not shown).

The Arg-finger residues are of particular interest as they reach across subunits and contact ATP that is bound to the neighboring subunit (Ogura et al., 2004). In Hsp104, the predicted Arg-fingers are Arg333/Arg334 and Arg765 (Fig. 1A and S1). Consistent with their known function in other AAA+ machines (Karata et al., 1999; Mogk et al., 2003; Werbeck et al., 2011; Yamasaki et al., 2011), these residues are found at the subunit interface in the Hsp104 hexamer (Fig. 1B and C) (Lee et al., 2010). The latter suggests a functional role of the predicted Arg-fingers in ATP hydrolysis as previously shown biochemically for bacterial ClpB (Mogk et al., 2003; Werbeck et al., 2011; Yamasaki et al., 2011). However, based on a

different Hsp104 hexamer model (Wendler et al., 2007; Wendler et al., 2009), it was argued that Arg333/Arg334 and Arg765, which were proposed to be located on the outside of the Hsp104 hexamer, cannot function as Arg-fingers in Hsp104 (Wendler et al., 2007). In this 'atypical' Hsp104 hexamer model, the M-domains are either in the interior or intercalated between subunits (Wendler et al., 2007; Wendler et al., 2009). As a consequence, it was proposed that arginines in the M-domain may assume the role of the Arg-fingers instead of the predicted Arg-finger residues.

Here, we employed biochemical approaches using engineered Hsp104 variants that featured mutations in the Arg-finger and Walker B motifs of Hsp104 to address the role of these conserved structural elements for Hsp104 function. Notably, mutations of the Walker B glutamates impact the ATPase and chaperone activities in a manner dependent on the chemical nature of the mutation. More importantly, we found that the predicted Arg-finger residues Arg333/Arg334 and Arg765 are essential for the ATPase and chaperone activities of Hsp104, consistent with their location in the 3D structure (Lee et al., 2010). Taken together, we conclude that conserved *cis*- and *trans*-acting elements common to diverse AAA+ machines regulate intra- and inter-ring communication in Hsp104, which is essential for its chaperone function.

Materials and Methods

Cloning and site directed mutagenesis

A total of 26 unique Hsp104 variants were made and analyzed in this study. All Hsp104 mutants were generated by site-directed mutagenesis from wild-type Hsp104 (Hsp104/pProEx HTb) (Lee et al., 2010) to generate TEV protease-cleavable, N-terminal His₆-tagged variants (Table S1). Generated variants include the single Walker B mutants Hsp104_{E285Q} (Q1), Hsp104_{E687Q} (Q2), Hsp104_{E285A} (B1), and Hsp104_{E687A} (B2), the Arg-finger mutants Hsp104_{R333A} (R1a), Hsp104_{R334A} (R1b), and Hsp104_{R765A} (R2), and the M-domain mutants Hsp104_{R419M} (R419M), Hsp104_{R419A} (R419A), Hsp104_{R444M} (R444M), Hsp104_{R444A} (R444A), Hsp104_{R444K} (R444K), Hsp104_{R495M} (R495M), and Hsp104_{R495A} (R495A). All other Hsp104 variants (B1·B2, R1a·R1b, R1a·R2, R1b·R2, R1a·B1, R1b·B1, R1a·R1b·B2, R2·B1, R2·B2, R419M·B2, R444M·B1, R444M·B2) contain a combination of two or three substitutions in either the Walker B glutamates, predicted Arg-fingers, and/or M-domain arginines. In cases where multiple point mutations were introduced, mutagenesis was carried out stepwise. Clones were fully sequenced to verify the presence of the desired mutations and to ensure that no inadvertent mutation was introduced.

Human Hsp70 (HSPA1A) was cloned by PCR from Addgene plasmid 19483 (Hageman and Kampinga, 2009) into pProEx HTb to generate an N-terminal His₆-tagged Hsp70. Enhanced GFP was cloned into a modified pET-21a plasmid (Novagen), which featured a TEV cleavable N-terminal His₇-tag and, additionally, the first 15 amino acids of RepA at the C-terminus (EGFP).

Protein expression and purification

Plasmids bearing wild-type or mutant Hsp104 were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). For each construct, a single colony was inoculated into 100 mL of LB media supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. Cultures were grown at 37°C to mid-log phase, induced with 0.5 mM IPTG, and allowed to grow for another 6 hours at 25°C. Cells were harvested, resuspended in 10 mL buffer A (25 mM Tris-HCl pH 7.5, 300 mM NaCl, 30 mM imidazole, 5% glycerol, 5 mM β-mercaptoethanol (βME)), and lysed by sonication on ice for 1 min in 10 sec bursts. The cleared lysate was applied to a 1 mL Ni-NTA agarose (Qiagen) column by gravity flow.

The column was washed with 100 mL buffer A, and bound proteins were eluted in 3.5 mL buffer A containing 300 mM imidazole. His-TEV protease was added to the eluate to cleave off the His-tag from Hsp104 variants. The cleaved product was diluted 10-fold with buffer B (25 mM Tris-HCl pH 7.5, 100 mM NaCl, 5% glycerol, 5 mM β ME) and re-applied to a 1 mL Ni-NTA agarose column to remove the His-TEV protease, the liberated His₆-tag, and any uncleaved protein. The flow-through was collected, concentrated and used in subsequent biochemical assays.

Protein expression and purification of human Hsp70 was adapted from a published protocol (Bhangoo et al., 2007). Briefly, Hsp70 was expressed in BL21-CodonPlus (DE3)-RIL cells. His-tagged Hsp70 protein was purified on a Ni-sepharose (GE Healthcare) column followed by anion-exchange chromatography. Yeast Hsp40 (Ydj1) was purified essentially as described (Lee et al., 2010).

EGFP was overexpressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells. Protein expression was induced at mid-log phase with 0.5 mM IPTG overnight at 16°C. Cells were resuspended in buffer A and lysed using a microfluidizer processor (Microfluidics). After removing cell debris, the supernatant was loaded onto a Ni-sepharose (GE Healthcare) column. The column was washed extensively with buffer A, and bound proteins were eluted over a linear gradient of buffer A containing 300 mM imidazole.

Analytical size-exclusion chromatography

Wild-type Hsp104 and the R1a, R1b, R2, R1a·R2 and R1b·R2 mutants (15 μ M) were analyzed on a Superdex 200 (10/30) column (GE Healthcare) pre-equilibrated in buffer C (50 mM MOPS-NaOH pH 7.5, 80 mM KCl, 6 mM β ME, 10 mM MgCl₂, 0.5 mM ATP). Protein concentration in each fraction was determined using the method by Bradford (Bradford, 1976), and plotted as a function of elution volume. To determine whether the mutants assembled into hexamers at the protein concentration used in our protein disaggregation assay (0.5 μ M), we repeated the same analytical size-exclusion chromatography experiment with and without ATP. Fractions were analyzed by Western blot using a polyclonal antibody against ClpB that cross-reacts with Hsp104.

Glutaraldehyde crosslinking

Wild type Hsp104 and mutants were diluted to either 0.5 μ M or 1.0 μ M in Buffer D (25 mM HEPES pH 7.5, 150 mM KAc, 10 mM Mg(Ac)₂, 10 mM DTT) supplemented with or without 5 mM ATP. Glutaraldehyde was added to a final concentration of 0.1% and crosslinking was performed for 10 min at 30°C. The crosslinking reaction was quenched by adding 1 M Tris-HCl (pH 7.5) to a final concentration of 0.1 M. The product was analyzed immediately on 3–8% Tris-Acetate-SDS PAGE gel (Invitrogen) and visualized by coomassie-blue staining.

ATPase activity assay

The ATPase activities of wild-type and mutant Hsp104 were measured at 30°C in the presence of a coupled ATP-regenerating system in buffer E (40 mM MOPS-NaOH pH 7.5, 150 mM KCl, 10 mM MgCl₂) essentially as described (Schaupp et al., 2007). The coupled regenerating system consisted of 10 mM ATP, 0.25 mM NADH, 0.5 mM phosphoenolpyruvate, 25 U/mL pyruvate kinase, and 85 U/mL lactate dehydrogenase (all from Roche).

Luciferase reactivation assay

10 μ M of FFL (Promega) was denatured for 30 min at 23°C in 7 M Urea and buffer D. The coupled chaperone assay was performed at 30°C by incubating 0.08 μ M of denatured FFL

with 1 μM (monomer) of Hsp104 or its mutant variant, 2 μM human Hsp70, and 1 μM yeast Hsp40 along with an ATP regenerating system consisting of 5 mM ATP, 20 mM phosphoenolpyruvate, and 2 μM pyruvate kinase over 120 min. Recovered FFL activity was measured every 20 min as previously described (Lee et al., 2010).

Disaggregation assay with β -galactosidase

β -galactosidase aggregates were prepared by incubating 0.4 μM of β -galactosidase (Sigma-Aldrich) in buffer D for 31 min at 60 °C. For the assay, 0.2 μM of heat-aggregated β -galactosidase was added to buffer D containing 1 μM (monomer) of the indicated molecular chaperones and the aforementioned ATP regenerating system. The coupled chaperone assay was performed at 30°C over 360 min. Recovered β -galactosidase activity was measured every 60 min as previously described (Lee et al., 2010).

Disaggregation assay with EGFP

His-tagged EGFP was used as a third model substrate. Aggregated EGFP was prepared by heating 4.5 μM of EGFP for 10 min at 75°C in buffer D supplemented with 1 μM of Hsp70 and Ydj1 and subsequently frozen in liquid nitrogen and stored at -80°C until use. Heat-aggregated EGFP (0.225 μM) was added to buffer D containing equimolar concentrations (0.5 μM monomer) of the indicated chaperones and the aforementioned ATP regenerating system. The recovery of EGFP fluorescence at 25 °C was monitored in real time over 90 min using a LS55 fluorescence spectrometer (Perkin Elmer). Excitation and emission wavelength were 488 nm and 510 nm, respectively, with 10 nm band width filter sets.

Results

Functional analysis of Hsp104 Walker B mutant variants

To determine the functional roles of conserved motifs in the AAA-1 and AAA-2 domains of Hsp104, we generated Hsp104 variants in which the ATPase activity in one of the two AAA+ domains was inactivated. Mutations of the catalytic Walker B glutamates in both AAA+ domains are known to abolish the ATPase activity of Hsp104 (Bosl et al., 2005; Schaupp et al., 2007). This Hsp104 double Walker B mutant binds nucleotides, but does not hydrolyze ATP and, as a result, functions as a substrate trap (Bosl et al., 2005). On the other hand, Hsp104 variants that feature a Walker B mutation in only one AAA+ domain are partially active and cooperate with Hsp70/40 to recover functional protein from aggregates (Schaupp et al., 2007). Consequently, the ATPase activity observed in an Hsp104 variant that features a mutation of the Walker B glutamate in only one AAA+ domain can be attributed solely to the activity of the other AAA+ domain.

In yeast Hsp104, the Walker B glutamates are Glu285 in the first AAA+ domain (Fig. 1B), and Glu687 in the second AAA+ domain (Fig. 1C). It was previously reported that Hsp104_{E285Q} (Q1) turns over ATP 8.4-fold faster than wild-type Hsp104 when ATP was not limiting, while Hsp104_{E687Q} (Q2) retained near wild-type activity (Schaupp et al., 2007). We found that the Q1 mutant hydrolyzed ATP 1.4 times faster than wild-type Hsp104, although the Q2 mutant retained only 56% of wild-type activity in our hands (Fig. 2A). The basis of this discrepancy is currently unknown.

Unexpectedly, we found that Hsp104_{E285A} (B1), which featured an alanine substitution instead of a glutamine mutation, displayed substantially reduced ATPase activities compared to wild-type Hsp104 (Fig. 2A). The B1 mutant turned over $6.7 \pm 0.5 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$, or 21% of wild-type Hsp104 level, whereas the B2 mutant turned over ATP with similar rates as the Q2 mutant, and retained 51% of wild-type Hsp104 activity (Fig. 2A). As anticipated, the B1·B2 double mutant did not hydrolyze ATP (Fig. 2A).

To determine whether the altered ATPase activities correlated with a net gain or loss of chaperone function, we assayed single Walker B mutants using a coupled chaperone assay with either chemically denatured FFL (Fig. 2B) or heat-aggregated β -galactosidase as substrate (Fig. 2C). Notably, Walker B mutants, regardless of the nature of the mutation or the amount of ATPase activity retained, did not recover as much functional protein from aggregates as did wild-type Hsp104 (Fig. 2B and C). The maximum recovered enzymatic activity was observed with the B1 mutant (73% for FFL and 21% for β -galactosidase), which had the lowest ATPase activity (21% of wild-type level). Recovery of enzymatic activity was most severely affected by the Q1 mutant (34% for FFL and 7% for β -galactosidase), which had the highest ATPase activity (145% of wild-type level). Thus, our findings suggest that a higher ATPase activity of Hsp104 mutants does not correlate with increased chaperone function.

Arginine fingers are required for inter- and intra-ring regulation of ATP hydrolysis

The Arg-finger residue is an essential *trans*-acting element in AAA+ ATPases, which reaches across the subunit interface to contact the bound nucleotide in the neighboring subunit (Karata et al., 1999; Ogura et al., 2004), and is readily identified by multiple-sequence alignment (Beyer, 1997; Iyer et al., 2004; Neuwald et al., 1999). In Hsp104, the predicted Arg-fingers are Arg333 and/or Arg334 in AAA-1 (Fig. 1B), and Arg765 in AAA-2 (Fig. 1C). Notably, the predicted Arg-finger residues are strictly conserved in all Hsp104/ClpB members (Fig. 1A and S1) and, in the case of ClpB, were shown to be crucial for ATP hydrolysis (Mogk et al., 2003; Werbeck et al., 2011; Yamasaki et al., 2011), consistent with the ClpB hexamer structure (Lee et al., 2007; Lee et al., 2003).

However, based on the fitted cryoEM structure of an atypical Hsp104 hexamer, it was proposed that arginines found in the non-conserved M-domain, but not the predicted Arg-fingers, serve the role of the critical *trans*-acting element in Hsp104 (Wendler et al., 2007). To examine the role of the predicted Arg-fingers, we generated six Hsp104 variants in which the predicted Arg-fingers were mutated in one or both AAA+ domains: Hsp104_{R333A} (R1a), Hsp104_{R334A} (R1b), Hsp104_{R765A} (R2), Hsp104_{R333A/R334A} (R1a·R1b), Hsp104_{R333A/R765A} (R1a·R2), and Hsp104_{R334A/R765A} (R1b·R2). Since Arg-finger residues have been proposed to stabilize the oligomeric assembly of AAA+ machines (Mogk et al., 2003; Song et al., 2000; Wang et al., 2005; Yamasaki et al., 2011), a state required for ATP hydrolysis, we first analyzed our mutants by analytical size-exclusion chromatography (Fig. 3A and B) and glutaraldehyde crosslinking (Fig. 3C, S2, and S3>). Notably, we found that Hsp104 variants featuring a mutation in one or both Arg-fingers formed stable hexamers in the presence of ATP at both high (15 μ M) and low protein concentrations (0.5 and 1.0 μ M) (Fig. 3A-C, S2 and S3).

Next, we examined whether Arg-finger mutations affect the ATPase activities of Hsp104. Strikingly, we found that substituting Arg333 (R1a), Arg334 (R1b), or both (R1a·R1b) with alanine essentially abolished the ATPase activities of Hsp104 (Fig. 3D). Notably, increasing the protein concentration of R1a and R1b did not increase the ATPase activities, suggesting that the remaining ATPase activity is negligible (Fig. S4). Consistent with this, combining Arg-finger mutations with mutations of the Walker B glutamate in AAA-1 (R1a·B1 and R1b·B1) or AAA-2 (R1a·R1b·B2) produced mutants that did not hydrolyze ATP (Fig. 3D). Consequently, double mutants with Arg-finger substitutions in both AAA+ modules (R1a·R2 and R1b·R2) lacked ATPase activity as well (Fig. 3D).

On the other hand, we found that the R765A mutation (R2) retained ATPase activity. The R2 mutant turned over ATP at a rate of $28.0 \pm 1.0 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$, or 87% of wild-type Hsp104 level. Notably, this activity was significantly faster than either single Walker B mutants (Fig. 3D, compare R2 with B1 and B2). Perhaps even more

surprisingly, the R2•B2 mutant, in which the R765A mutation was combined with the E687A mutation of the AAA-2 Walker B glutamate, is even more active as an ATPase than wild-type Hsp104, turning over $36.9 \pm 2.1 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$. To confirm that Arg765 is the Arg-finger in AAA-2, we generated the R2•B1 double mutant by combining the Hsp104_{R765A} variant with the E285A mutation in the first Walker B motif, which inactivates the ATPase activity of the AAA-1 ring. We found that the R2•B1 double mutation essentially abolished the ATPase activities of Hsp104 (Fig. 3D), suggesting that Arg765 is required to hydrolyze ATP in AAA-2. Increasing the protein concentration of the R2•B1 mutant did not increase the ATPase activity (Fig. S4).

Next, we examined the functional activity of the Arg-finger mutants using a coupled-chaperone assay. We found that all R1a and R1b-containing Hsp104 variants, which were ATPase-inactive (Fig. 3D), also had negligible chaperone activity (Fig. 3E and F). This was also the case for the R2 and R2•B2 mutants, which had substantial ATPase activity (Fig. 3D), but were functionally impaired (Fig. 3E and F, and S5). R2•B1, which had negligible ATPase activity (Fig. S4), also did not retain chaperone activity (Fig. 3E and F, and S5).

M-domain arginine mutants impact the ATPase and chaperone activities of Hsp104

It was proposed that the Hsp104 M-domain arginines, Arg419, Arg444, and Arg495, may assume the role of the predicted Arg-fingers (Wendler et al., 2007). If correct, such function would be surprising, since most Hsp100 proteins do not feature an M-domain, yet are functionally active ATPases. In addition, multiple-sequence alignment of Hsp104 homologs showed that the M-domain arginines are not conserved across Hsp104 proteins (Fig. 1A), with Arg444 being perhaps the sole exception. In bacterial ClpC, which features a shorter M-domain, Arg444 is conservatively substituted for lysine. Nevertheless, if M-domain arginines indeed assume the role of the Arg-fingers, we would expect to find that mutations of these arginines would abolish Hsp104 ATPase activity, as shown for the R1a, R1b, and R2•B1 mutants (Fig. 3D and S4).

To examine the role of M-domain arginines, we generated and determined the ATPase activities of Hsp104_{R419M} (R419M), Hsp104_{R444M} (R444M), and Hsp104_{R495M} (R495M). Notably, we found that a mutation of either Arg419 or Arg444 to methionine reduced the ATPase activity to 29% and 50% of wild-type Hsp104, respectively (Fig. 4A), while the R495M mutation increased ATPase turnover to $378.5 \pm 14.5 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$, representing a nearly 12-fold increase over wild-type Hsp104 rates (Fig. 4A).

To directly compare M-domain arginine mutations with our R1a, R1b, and R2 mutants, we substituted Arg419, Arg444, and Arg495 with alanine to generate Hsp104_{R419A} (R419A), Hsp104_{R444A} (R444A), and Hsp104_{R495A} (R495A). We found that the alanine mutants had similar ATPase activities as the arginine to methionine mutants (Fig. 4A). For instance, Hsp104 featuring an R419A mutation hydrolyzed $14.3 \pm 0.2 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$, or 44% of wild-type Hsp104 level, and the R444A mutation retained 55% of wild-type Hsp104 ATPase activity (Fig. 4A). Similarly, the R495A mutation increased ATP turnover 12-fold, to $390.1 \pm 5.7 \mu\text{mol ATP} (\mu\text{mol Hsp104})^{-1} \text{ min}^{-1}$. Consequently, the increased ATPase activity observed for both Arg495 substitutions strongly supports the notion that Arg495 cannot be the Arg-finger. Finally, because Arg444 is conservatively substituted with lysine in ClpC (Fig. 4A), we also generated and analyzed the Hsp104_{R444K} (R444K) mutant, and found that it had a similarly impaired ATPase activity as the methionine and alanine mutants (Fig. 4A).

The Arg419 and Arg444 mutants retained partial chaperone activity (Fig. 4B and C). We found that the R419M and R419A mutants recovered 63% and 71% of FFL activity, respectively, relative to wild-type Hsp104 (Fig. 4B). The R444M, R444A and R444K

mutants recovered 63%, 67% and 54% of FFL relative to wild-type Hsp104 level (Fig. 4B). Similar results were also obtained with heat-aggregated EGFP, which, in general, gave somewhat lower recovery yields for each mutant (Fig. 4C).

Since mutations of Arg419 and Arg444 yielded Hsp104 variants with reduced ATPase activities and diminished function in thermotolerance development (Wendler et al., 2007), it was speculated that Arg419 might assume the role of the Arg-finger for AAA-1, and Arg444 for either AAA-1 or AAA-2 (Wendler et al., 2007). To test the role of Arg419 and Arg444 in ATP hydrolysis, we generated M-domain arginine mutations in combination with mutations in the Walker B glutamate of either AAA-1 and/or AAA-2. Importantly, we found that when the R419M and R444M were combined with either the B1 or B2 mutation, the resulting R419M·B2, R444M·B1, and R444M·B2 double mutants had reduced but measurable ATPase and chaperone activities compared to wild-type Hsp104 (Fig. 5A-C). Notably, the remaining chaperone activities (Fig. 5B and C) were significantly higher than those observed with the analogous Walker B mutants featuring an additional mutation in the predicted Arg-finger residues (Fig. 3E and F)

Discussion

A hallmark of AAA+ machines is the presence of common *cis* and *trans*-acting structural elements and motifs, which are essential for ATP-fueled mechanical work. Here, we conducted a detailed and extensive biochemical analysis of the *trans*-acting Arg-finger residues in Hsp104. Our findings confirm the role of the predicted Arg-finger residues as key *trans*-acting elements required for ATP hydrolysis within each of the two AAA+ rings, which is consistent with the conserved hexamer structure of Hsp104 (Lee et al., 2010) but incompatible with the proposed atypical hexamer model (Wendler et al., 2007). Lack of ATPase activities in Hsp104 mutants bearing alanine substitutions in the AAA-1 Arg-fingers, alone or in combination with other mutations, clearly indicate that Arg333 and Arg334 are absolutely required for ATP hydrolysis and chaperone function. While it remains unclear why there are two Arg-fingers in AAA-1, both residues are essential and neither can replace the other. The requirement for the Arg-finger in AAA-2 was demonstrated by the inability of the R2·B1 double mutant to turn over ATP. The lack of functional activity was not due to an oligomerization defect, since Arg-finger mutants retained the ability to form hexamers in the presence of ATP (Fig. 3A-C, S2 and S3).

Interestingly, our experiments also demonstrate that Arg-fingers not only regulate ATP hydrolysis in the proximal ring, but also regulate the ATPase activity in the distal ring. For instance, in the single R1a and R1b mutants, the ATPase activity was abolished, even though the AAA-2 domain should remain catalytically active. Moreover, an inter-ring regulatory function was demonstrated at least for the AAA-2 Arg-finger, because the R2·B2 double mutant hydrolyzed ATP faster than wild-type Hsp104, and substantially faster than either the single R2 or B2 mutants alone.

On the other hand, mutational analysis of M-domain arginines indicate that these residues are not strictly required for ATP hydrolysis, but rather affect the Hsp104 ATPase and chaperone activities in a complex manner. Mutations of Arg495 strongly stimulated the Hsp104 ATPase activity (Fig. 4A), while equivalent mutations of Arg444 and Arg419 resulted in a net loss in both ATPase and chaperone activities (Fig. 4A-C). The impaired function caused by the Arg444 and Arg419 mutations is not entirely unexpected, as several studies have documented the functional importance of the M-domain and its sensitivity to mutations. For instance, single point mutations in the Hsp104 M-domain have a profound effect on yeast cell morphology, growth, and thermotolerance (Schirmer et al., 2004). Moreover, mutations in the M-domain of ClpB, the bacterial ortholog of Hsp104, have been

shown to impair ATPase (Kedzierska et al., 2003) and protein disaggregation activity (Haslberger et al., 2007). Taken together, our results suggest that the M-domain arginines do not function as arginine fingers, but likely affect the communication pathway between the AAA-1 and AAA-2 domains.

In addition, our results also reveal finer details of the activity and interaction of AAA+ domains. First, our results imply that, in addition to its role in ATP hydrolysis, the Walker B glutamate of AAA-1 may have an additional function in inter-subunit regulation of the ATPase activity in Hsp104. Previously, it was shown that the Walker B mutation in the AAA-1 domain keep it in the ATP-bound state and stimulates ATP hydrolysis in the AAA-2 ring. Here, we show that the hydrolytically active AAA-2 ring responds differently depending on the chemical nature of the Walker B mutation. Second, our results indicate that while mutations of either the Arg-finger or the Walker B glutamate result in impairment of ATP hydrolysis in the proximal ring, their functional consequences in the distal ring are different. For example, when the Walker B glutamate in AAA-1 is mutated to glutamine, ATP hydrolysis in AAA-2 was stimulated (Fig. 2A), whereas when the Arg-fingers (R1a, R1b, and R1a•R1b) in AAA-1 are mutated, ATP hydrolysis in AAA-2 was abolished (Fig. 3D).

Conclusion

Our results are consistent with the notion that ATP hydrolysis within each AAA ring strictly requires the functionally conserved Walker B motif and the predicted Arg-finger, as observed for other AAA+ machines. Consequently, our results are fully compatible with the fitted cryoEM structures of Hsp104 and ClpB (Lee et al., 2007; Lee et al., 2010; Lee et al., 2003) but incompatible with the proposed model of an atypical Hsp104 hexamer (DeSantis and Shorter, 2012 Wendler et al., 2007; Wendler et al., 2009). Finally, our work also reinforces the notion that measuring ATPase activities alone is not an absolute measure of Hsp104 function and needs to be interpreted with caution, although lack of ATPase activity always correlates with loss of chaperone function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Conserved structural elements in the Hsp104 AAA+ ATPase. (A) Multiple sequence alignment of yeast Hsp104, plant Hsp101, bacterial ClpB, and related Hsp100 proteins. Sc, *S. cerevisiae*; At, *A. thaliana*; Ec, *E. coli*; Tt, *T. thermophilus*; Vc, *Vibrio cholerae*; Sa, *S. aureus*; Mb, *M. burtonii*; Se, *S. enterica*; Bs, *B. subtilis*; Mt, *M. tuberculosis*; Ba, *B. animalis*. The numbering refers to the Hsp104 sequence. Strictly conserved residues are highlighted magenta, and residues conserved with yeast Hsp104 are shaded yellow. Arg333/Arg334 and Arg765 are marked with diamonds, and M-domain arginines (Wendler et al., 2007) are marked with “#” and are boxed. The catalytic Walker B glutamates are marked

with “+”. Top down view of **(B)** the AAA-1 and **(C)** the AAA-2 ring of the Hsp104 homo-hexamer based on the atomic structure fit of the single-particle cryoEM reconstruction (Lee et al., 2010). Two adjacent Hsp104 subunits are colored for clarity. The insets depict detailed views of the intersubunit interface in **(B)** AAA-1 and **(C)** AAA-2. Glu285 and Glu687 are the Walker B glutamates, and Arg333/Arg334, and Arg765 are the predicted Arg-finger residues in the AAA-1 and AAA-2 subdomains, respectively. The ATP-binding site is located at the intersubunit interface.

Figure 2.

Functional activities of Hsp104 and Hsp104 Walker B variants. Hsp104 single Walker B mutants in which the catalytic glutamate was replaced with either alanine (B1:Hsp104_{E285A}; B2:Hsp104_{E687A}) or glutamine (Q1:Hsp104_{E285Q}; Q2:Hsp104_{E687Q}). Standard deviations of three independent measurements are shown. **(A)** ATPase rates of Hsp104 (WT) and Hsp104 Walker B mutants determined at 30°C in the presence of a coupled ATP regenerating system. The Hsp104 double Walker B mutant (B1·B2:Hsp104_{E285A/E687A}) is an inactive ATPase. **(B-C)** Disaggregation of model substrates by Hsp104 and Hsp104 single Walker B variants in the presence of Hsp70/40. Recovered enzymatic activities were

determined at 30°C and are expressed relative to the recovered activity by the wild-type bi-chaperone system. The inset shows a time course analysis, with the recovered enzymatic activities shown as percentage of the initial activity of substrate. Standard deviations of three independent measurements are shown. **(B)** Recovered activity from urea-denatured FFL at 120 min. **(C)** Activity recovered from heat-denatured β -galactosidase at 360 min.

Figure 3.

The Arg-finger residues, Arg333/Arg334 (R1a/R1b) and Arg765 (R2), are critical for Hsp104 function. **(A)** Size-exclusion chromatography analysis of the Hsp104 hexamer assembly (WT) and representative Arg-finger mutants Hsp104_{R333A} (R1a), Hsp104_{R334A} (R1b), Hsp104_{R765A} (R2), Hsp104_{R333A/R765A} (R1a•R2) and Hsp104_{R334A/R765A} (R1b•R2) at 15 μ M in the presence of 0.5 mM ATP. **(B)** Western blot analysis of fractions collected from size-exclusion chromatography analysis of wild type Hsp104, R1a, R1b, R2, R1a•R2 and R1b•R2 at 0.5 μ M with or without ATP. **(C)** Glutaraldehyde crosslinking of wild type Hsp104, R1a, R1b, R2, R1a•R2 and R1b•R2 at 0.5 μ M with or without ATP. **(D)** ATP turnover rates of Hsp104 (WT) and Hsp104 variants that carry a mutation in the Arg-finger, in the Walker B glutamate, or in both motifs. R1a: R333A; R1b:R334A; R1a•R1b:R333A/R334A; R1a•R2:R333A/R765A; R1b•R2:R334A/R765A; R1a•B1:R333A/E285A; R1b•B1:R334A/E285A; R1a•R1b•B2:R333A/R334A/E687A; R2:R765A; R2•B2:R765A/E687A; R2•B1:R765A/E285A; B1:E285A; B2:E687A. Standard deviations of three

independent measurements are shown. **(E-F)** Disaggregation of model substrates by Hsp104 and the aforementioned Hsp104 variants in the presence of Hsp70/40 and an ATP regenerating system. Standard deviations of three independent measurements are shown. **(E)** Urea-denatured FFL and **(F)** heat-aggregated β -galactosidase were reactivated at 30 °C for 120 and 360 min, respectively. Recovered enzymatic activities are shown relative to the recovered yield by wild-type Hsp104 in the presence of Hsp70/40.

Figure 4.

M-domain arginine mutations affect Hsp104 function in a complex manner. **(A)** ATPase of Hsp104 and Hsp104 variants featuring a mutation of Arg419, Arg444 or Arg495. **(B-C)** Chaperone activities of Hsp104 and Hsp104 variants featuring a mutation of Arg419 or Arg444. Recovered enzymatic activities are expressed relative to the recovered activity by the wild-type bi-chaperone system. **(B)** Enzymatic activities recovered from urea-denatured FFL at 120 min. The inset shows a time course analysis with the recovered enzymatic activities shown as percentage of the initial activity of substrate. Standard deviations of three independent measurements are shown. **(C)** Recovered fluorescence from heat-aggregated

EGFP at 90 min. Data from three experiments are shown. The inset shows the real-time fluorescence data from a representative experiment.

Figure 5.

M-domain arginines do not act as Arg-fingers. Standard deviations of three independent measurements are shown. **(A)** ATP hydrolysis rates of Hsp104 and Hsp104 variants that feature an R419M or R444M mutation in combination with the Walker B mutation E285A (B1) or E687A (B2). **(B-C)** Protein disaggregation of aggregated model substrates by Hsp104 and M-domain mutants in the presence of Hsp70/40 and an ATP regenerating system. Recovered enzymatic activities are expressed relative to the recovered activity by the wild-type bi-chaperone system. The insets show a time course analysis, with the recovered enzymatic activities shown as percentage of the initial activity of substrate. **(B)**

Recovered enzymatic activities from urea-denatured FFL at 120 min. (C) Enzymatic activities recovered from heat-aggregated β -galactosidase at 360 min.