Defining a Pathway of Communication from the C-Terminal Peptide Binding Domain to the N-Terminal ATPase Domain in a AAA Protein

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Summary

AAA proteins remodel other proteins to affect a multitude of biological processes. Their power to remodel substrates must lie in their capacity to couple substrate binding to conformational changes via cycles of nucleotide binding and hydrolysis, but these relationships have not yet been deciphered for any member. We report that when one AAA protein, Hsp104, engages polypeptide at the C-terminal peptide-binding region, the ATPase cycle of the C-terminal nucleotide-binding domain (NBD2) drives a conformational change in the middle region. This, in turn, drives ATP hydrolysis in the N-terminal ATPase domain (NBD1). This interdomain communication pathway can be blocked by mutation in the middle region or bypassed by antibodies that bind there, demonstrating the crucial role this region plays in transducing signals from one end of the molecule to the other.

Introduction

The AAA proteins are <u>A</u>TPases <u>a</u>ssociated with various cellular <u>a</u>ctivities, including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication, and intracellular motility (Neuwald et al., 1999; Vale, 2000; Ogura and Wilkinson, 2001). They have a remarkable capacity to drive the assembly and disassembly of protein complexes. For example, NSF governs membrane fusions by disassembling SNARE complexes (Rothman, 1994). Two AAA proteins (γ and δ') in *Escherichia coli* clamp loader complex open the sliding clamp ring and load it onto DNA to enable the processive motion of the DNA polymerase (Turner et al., 1999). Other AAA proteins remodel substrates to facilitate proteolysis (Walz et al., 1998; Weber-Ban et al., 1999; Kim et al., 2000; Singh et al., 2000).

All of the characterized family members are oligomeric. Each AAA module contains a highly conserved nucleotide binding domain (NBD) and a second domain that may confer functional specificity (Neuwald et al.,

1999). The ATP binding and hydrolysis cycles of these molecular machines presumably couple substrate binding to conformational changes and thereby provide the capacity to remodel substrates. The mechanism by which this coupling is affected has yet to be defined for any AAA protein.

Hsp104, a member of the HSP100/Clp subfamily of AAA proteins, is a major heat-shock protein in the yeast, *Saccharomyces cerevisiae*. It has two unusual protein remodeling functions, underscoring the wide diversity of biological functions among AAA proteins. After exposure to severe environmental stress, Hsp104 disassembles protein aggregates formed from denatured proteins and thereby increases survival as much as 10,000-fold (Sanchez and Lindquist, 1990; Parsell et al., 1991; Sanchez et al., 1992; Parsell et al., 1994b; Glover and Lindquist, 1998). At normal temperatures, the conformational remodeling activity of Hsp104 controls the inheritance of several protein-based genetic elements (prions), including [*PSI*+] (Chernoff et al., 1995), [*RNQ*+] (Sondheimer and Lindquist, 2000), and [*URE3*] (Moriyama et al., 2000).

As is typical of members of the type II family of AAA proteins, Hsp104 contains two highly conserved nucleotide binding domains (NBD) (Schirmer et al., 1996). The NBDs are flanked by less-conserved N-terminal, "middle," and C-terminal regions. In related proteins, the N- and C-terminal regions appear to bind substrates and/or effector proteins (Smith et al., 1999; Barnett et al., 2000; Lo et al., 2001). The middle region is the most variable and least understood. A recent alignment of Hsp104 (J.R.G., unpublished data) suggests the middle region represents an insertion between the Rossmann fold and the putative sensor 2 motif of the first NBD. In HslU, the AAA module has an insertion, the I domain, in a different position of the NBD (Bochtler et al., 2000; Sousa et al., 2000). Both NBDs are essential for function: substitution of the crucial lysine residue in the Walker-A site by threonine in either NBD eliminates thermotolerance (Parsell et al., 1994a) and interferes with prion maintenance (Chernoff et al., 1995). The basal ATPase activity of Hsp104 in vitro is primarily attributable to the N-terminal NBD (NBD1) (Parsell et al., 1994a; Schirmer et al., 1998; Hattendorf and Lindquist, 2002a).

Attempts to define the effects of substrate binding on the ATPase activity and the conformations of Hsp104's various domains have been thwarted by three problems. First, its substrates are aggregated or aggregation-prone proteins, which are inherently difficult to manipulate. Second, the domains of Hsp104 have a high degree of conformational flexibility relative to each other (as revealed by electron microscopy [Parsell et al., 1994a]), and it has not been possible to stabilize individual conformations with nucleotides or their analogs (A. Kowal and S.L.L. unpublished data). Third, Hsp104's interactions with its substrates are transient and are not stabilized by nucleotide analogs. For example, although Hsp104 resolubilizes heat-induced protein aggregates (Parsell et al., 1994b) and is localized around these aggregates in vivo (Fujita et al., 1998; Kawai et al., 1999),

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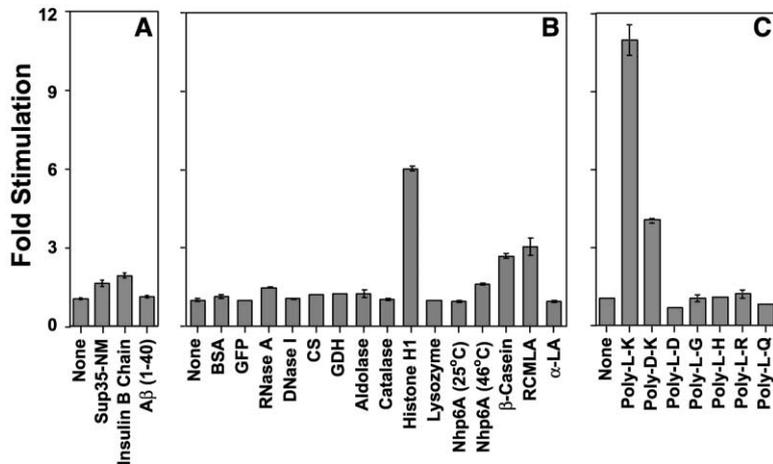
Figure 1. Structurally Flexible and Lysine-Rich Polypeptides Stimulate the ATPase Activity of Hsp104

The values shown are the average fold stimulations relative to the ATPase activity in the absence of added proteins or polypeptides, as observed in at least three independent experiments. (A) Amyloidogenic polypeptides; (B) proteins with different structures; (C) synthetic polypeptides of comparable chain lengths (range 30–50 kDa).

aggregates isolated from heat-shocked cells by several methods do not retain Hsp104 (A.G.C. and S.L.L., unpublished data). And, although purified Hsp104 affects the conformational state of prion peptides in vitro (Deb-Burman et al., 1997; Schirmer and Lindquist, 1997), stable cocomplexes have yet to be isolated. To circumvent these problems, we sought soluble model polypeptides that might form stable specific interactions with Hsp104. Here we show that poly-L-lysine provides such an interaction, binding Hsp104 in a length-dependent and highly cooperative manner. This interaction stabilized Hsp104 in an active state allowing us to apply a wide variety of methods to analyze the dynamic consequences of the interaction. Other approaches were then employed to establish a sequential pathway of communication from one domain to another, providing a model for the reaction mechanism of Hsp104 and a potential framework for the reaction mechanisms of other members of this diverse family.

Results

Identification of Peptides that Stimulate the ATPase Activity of Hsp104

To date, the tested substrates and peptides that are remodeled by Hsp104 and the cofactors that promote its function interact with the protein transiently and stimulate or inhibit ATP hydrolysis less than 2-fold (Schirmer and Lindquist, 1997). Similarly, we found that the first 253 amino acids of Sup35, which are sufficient to transfer Hsp104-dependent conformational conversion activity to another protein (Li and Lindquist, 2000), stimulated the ATPase activity of Hsp104 but only 1.5-fold (Figure 1A). Other amyloidogenic proteins that change conformation in the presence of Hsp104 (Schirmer and Lindquist, 1997) were also only marginally stimulatory (Figure 1A). As in earlier studies, none of these formed stable complexes with Hsp104, and numerous attempts to stabilize Hsp104:substrate interactions with various nucleotides and their analogs failed (data not shown). Since the free-running ATPase activity of Hsp104 is high ($k_{cat} \approx 200$ min^{-1}; (Schirmer et al., 1998), transiently interacting substrates might have little effect on total hydrolysis because the protein is occupied by substrate for only a small fraction of the sampling time. Therefore, to

identify molecules that might form more stable complexes with Hsp104, we tested the capacity of a wide variety of proteins and polypeptides to exert a stronger influence (inhibiting or activating) on the protein's ATPase activity.

The vast majority had no influence on Hsp104's ATPase activity (Figure 1B). These included proteins with diverse secondary, tertiary, and quaternary structures, including β barrel (GFP), α-helical (α-lactalbumin, citrate synthase, BSA), and mixed α/β (lysozyme, RNaseA, DNase1, aldolase, catalase, etc.) proteins, as well as monomeric (lysozyme), dimeric (citrate synthase, aldolase), and multimeric proteins (catalase, glutamic dehydrognase) (Figure 1B). Histone H1, a lysine-rich protein, stimulated Hsp104 ATPase activity ~6-fold. By NMR, roughly 50% of histone H1 polypeptide is unstructured when not bound to DNA (Hartman et al., 1977). Another lysine-rich protein, Nhp6A (a chromatin-associated protein), which unfolds at 39°C (Yen et al., 1998), was not stimulatory at 25°C, but reproducibly stimulated Hsp104's ATPase activity ~1.5 fold at 46°C (Figure 1B). An ariginine-rich, well-folded protein, lysozyme, was not stimulatory (Figure 1B). β-casein and RCMLA (reduced and carboxymethylated α-lactalbumin), which are conformationally flexible (Kuwajima, 1996), stimulated Hsp104 ATPase activity 2- to 3-fold (Figure 1B).

We also tested many synthetic polyamino acids. Most of these were not stimulatory. However, poly-L-lysine (pK^L), which has a circular dichroism (CD) spectrum characteristic of random-coil structure (data not shown), stimulated ATPase activity very strongly (Figure 1C). Stimulation was not simply due to abundant positive charges, since poly-L-arginine was not stimulatory (Figure 1C). Moreover, stimulation was chirally selective: pK^L stimulated much more than poly-D-lysine (pK^D, Figure 1C). These and other data (see Discussion) suggest that lysine-rich regions and unstructured conformations are recognition factors for Hsp104.

The Importance of Cooperativity in ATP Stimulation by pK^L

As a hexameric protein remodeling factor, subunits of Hsp104 are likely to interact cooperatively with its substrates (or cofactors). If pK^L's interactions with Hsp104 mimic bona fide interactions, it should stimulate ATPase
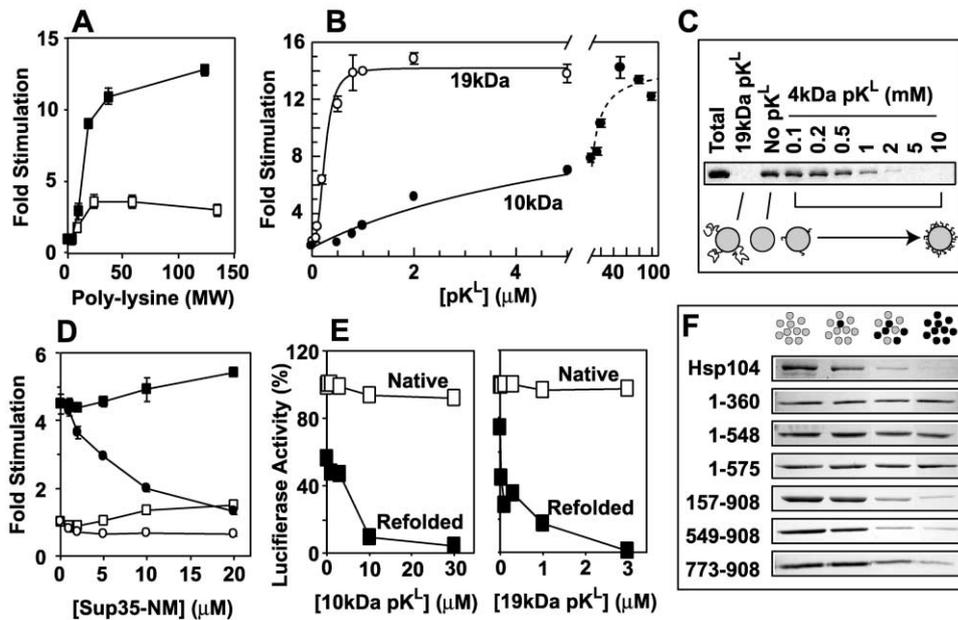
Figure 2. Hsp104 Binds to pK^L in a Highly Selective and Cooperative Manner

(A) Effect of chain length of pK^L (closed squares) and pK^D (open squares) on Hsp104 ATPase activity.

(B) Binding properties of 10 kDa (closed circles) and 19 kDa (open circles) pK^L to Hsp104. The K_ds were 5.7 ± 1.7 μM and 0.25 ± 0.02 μM and the Hill coefficients were 0.9 and 2.9 for 10 and 19 kDa pK^Ls, respectively.

(C) Coomassie-stained SDS-PAGE of the amount of Hsp104 in the supernatant after incubating with 4 kDa pK^L cross-linked to Sepharose beads at increasing packing density (see cartoon). 19 kDa pK^L cross-linked to beads at 0.1 mM efficiently binds Hsp104.

(D) Effect of various concentrations of unpolymerized (squares) and polymerized (circles) Sup35-NM on the ATPase activity of Hsp104 in the absence (open symbols) or presence (closed symbols) of 19 kDa pK^L. Control experiments established that pK^L did not bind to NM (data not shown).

(E) Effect of pK^L (left 10 kDa; right 19 kDa) on the Hsp104-dependent reactivation of aggregated firefly luciferase as described in Glover and Lindquist (1998) (closed squares). The 19 kDa pK^L was at least 10-fold more efficient than the 10 kDa pK^L in inhibiting substrate binding to Hsp104. The pK^Ls had no effect on the activity of native luciferase (open squares).

(F) Identification of CTD as the pK^L binding domain of Hsp104. Binding of fragments of Hsp104 (indicated on left) to various ratios of mock-treated and 19 kDa pK^L-coupled beads (10:0, 9:1, 5:5, and 0:10) was tested by SDS-PAGE of protein in the supernatant.

activity in a cooperative manner. First, we measured Hsp104 ATPase activity with poly-lysines of different average chain lengths (all with random-coil CD signals), keeping the total number of lysine residues in solution constant. Stimulation by poly-D-lysine did not increase as chain length increased; stimulation by poly-L-lysine was strongly dependent on chain length (Figure 2A).

Next, we utilized ATPase stimulation to measure the binding affinity of Hsp104 for pK^L and to probe the nature of this interaction. Short pK^Ls (avg 4 kDa) showed no stimulation at any concentration tested (range 0.001–10 mM, data not shown). Stimulation by 10 kDa pK^L indicated a K_d of ~6 μM and noncooperative binding (Figure 2B). The 19 kDa pK^L exhibited a K_d of 0.25 μM, and binding was strongly cooperative (Figure 2B) with a Hill coefficient of ~2.9. Histone H1 also bound Hsp104 cooperatively with a similar K_d (data not shown). For 37 kDa pK^L, binding was not cooperative and not specific to a particular domain of Hsp104 (data not shown) as it was with 19 kDa pK^L (see below). Since very long-chain pK^Ls show nonspecific interactions with some proteins (Panse et al., 2000), 37 kDa pK^L was not employed further. The strong difference in Hsp104 ATPase stimulation and binding affinity between 10 kDa and 19 kDa pK^L suggests that the larger molecule can simultaneously interact with multiple binding sites and thereby cooperatively stimulates ATPase activity.

The Relevance of Hsp104-pK^L Interaction

Hsp104 resolves protein aggregates and must be able to distinguish the soluble and the aggregated states of its substrates. Like folded proteins, aggregated proteins tend to bury hydrophobic residues and keep charged residues on the surface (Fink, 1998). However, aggregates of denatured proteins have a larger surface area than individual, folded proteins and would display less rigid polypeptide chains on their surfaces. Simultaneous binding of Hsp104 subunits to a large charged (especially lysine-rich) surface with some flexible regions might provide a basis for substrate recognition. If so, the 4 kDa pK^L, which shows no stimulation of Hsp104 ATPase activity even at thousands of times the concentration required for stimulation by 19 kDa pK^L, might be capable of interacting with Hsp104 if it were presented in a clustered, spatially restricted manner. Indeed when 4 kDa pK^L was cross-linked to Sepharose beads at different packing densities, beads with low densities failed to retain Hsp104, leaving most of it in solution; beads with high densities retained it (Figure 2C). The 19 kDa pK^L bound Hsp104 even when cross-linked to beads at the lowest density. Thus spatially clustered lysines on flexible polypeptides provide a selective recognition feature for Hsp104.

To investigate the biological relevance of pK^L:Hsp104 interaction, we tested the ability of a natural substrate
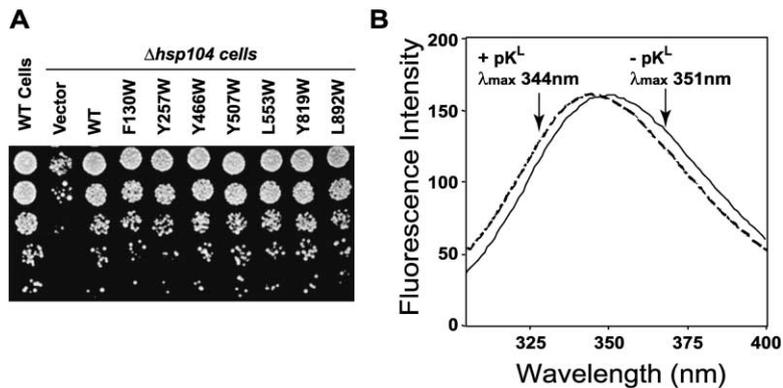
Figure 3. Analysis of Tryptophan Mutants of Hsp104

(A) Thermotolerance of Δhsp104 *S. cerevisiae* cells expressing wild-type Hsp104 or each tryptophan substitution mutant was determined by spotting 5-fold serial dilutions of heat-treated cells on rich medium. The mutants provide thermotolerance comparable to wild-type Hsp104.

(B) Tryptophan fluorescence spectra of the Hsp104 Y507W mutant in the presence (dashed line) and absence (solid line) of 19 kDa pK^L. A 7 nm blue shift in the wavelength of maximum fluorescence indicates a conformational change in the middle region.

of Hsp104 to interfere with pK^L-mediated stimulation of ATPase activity. In vivo, Hsp104 affects the conformational state of Sup35, which forms the protein-based genetic element (prion) known as [*PSI*+] (for review see Serio and Lindquist, 2000). The prion-determining region of Sup35 (NM) is sufficient for recognition by Hsp104 (Li and Lindquist, 2000) and contains a lysine-rich segment (23 lysines in 90 residues). In vitro, NM exists in two distinct conformational states—an unpolymerized, random-coil-rich state and a polymerized, β sheet-rich protein amyloid (Serio et al., 2000). Unpolymerized NM did not affect the pK^L-stimulated ATPase activity of Hsp104 (Figure 2D), although it had a mildly stimulatory effect on the basal ATPase activity (see also Figure 1A). The polymerized form of NM blocked pK^L-stimulated ATPase activity. Neither conformation bound to 19 kDa pK^L (data not shown). Thus, a bona fide Hsp104 substrate competes with pK^L for binding to Hsp104 in a conformationally specific manner.

The second known function of Hsp104 is in reactivating proteins from aggregates caused by heat shock (Parsell et al., 1994b). This function can be assayed in vitro by the Hsp104-dependent reactivation of aggregated firefly luciferase (Glover and Lindquist, 1998). Both 10 and 19 kDa pK^{L}s efficiently competed with aggregated firefly luciferase, leading to a dramatic decrease in the yield of active firefly luciferase (Figure 2E). This was specifically due to an effect on Hsp104 function because pK^L affected neither the activity of native luciferase (Figure 2E) nor of Hsp70 and Hsp40 (data not shown). Reflecting the nature of their interactions with Hsp104 (Figure 2B), 19 kDa pK^L interfered with luciferase reactivation much more efficiently than 10 kDa pK^L. Strong inhibition was achieved at equal concentrations (1 μM each; Figure 2E) of pK^L and Hsp104.

Identification of the pK^L Binding Domain in Hsp104

To identify the pK^L binding region in Hsp104, various fragments of Hsp104 were created. V8- and trypsin-sensitive sites were mapped in Hsp104 to roughly define domain boundaries and expression constructs for three prominent V8-resistant fragments, 1–360, 1–548 and 549–908, and a trypsin-resistant fragment, 157–908 (S. Lee, P. Sigler, and S.L.L., unpublished data), were produced. Since the fragment 1–548 lacked the sensor 2 motif of NBD1 an additional N-terminal fragment, 1–575

was produced encompassing the entire NBD1. The C-terminal boundary of the second AAA module of Hsp104 was mapped in the vicinity of residue 773 by modeling residues 549–908 against the crystal structure of the NSF-D2 domain (Hattendorf and Lindquist, 2002b). The C-terminal domain (CTD; residues 773–908) was also purified. All fragments produced CD spectra typical of well-folded proteins (data not shown).

Using the Sepharose bead assay, full-length Hsp104 was retained by beads cross-linked to 19 kDa pK^L but not by mock-treated beads (Figure 2F). Fragments containing only the N-terminal domain, NBD1, or the middle region (1–360, 1–548, and 1–575) showed no appreciable binding in any sample. The fragments containing the CTD (157–908 and 549–908) bound to the beads in the same manner as wild-type protein (Figure 2F). The CTD alone also bound pK^L, but the binding was weaker. Because this fragment is monomeric (by size exclusion chromatography; data not shown), weaker binding is presumably due to the fact that it cannot bind pK^L cooperatively. Notably, other members of the HSP100/Clp family (ClpA, ClpB, ClpX, and HslU) also bind substrates (or cofactors) via a similar C-terminal domain called the sensor and substrate discrimination (SSD) domain (Smith et al., 1999).

pK^L-Induced Conformational Change in the Middle Region Revealed by Tryptophan Substitution Mutants

The stability of pK^L binding to Hsp104 allowed us to test the consequences of CTD engagement with polypeptides on the rest of Hsp104 using methods that could not otherwise be employed. First, we took advantage of the fact that tryptophan fluorescence presents a sensitive probe for conformational change and the fact that wild-type Hsp104 does not contain any tryptophan residues. A series of single tryptophan substitution mutations was created throughout the Hsp104 sequence, individually replacing hydrophobic residues F130, Y257, Y466, Y507, L553, Y819, and L892. Each substitution mutant provided full thermotolerance to yeast cells with a disrupted *HSP104* gene (Figure 3A). When purified, each mutant exhibited similar CD spectra and the same pK^L-stimulated ATPase activity as the wild-type protein (data not shown).

Wild-type Hsp104 showed no tryptophan fluorescence, indicating that other tryptophan-containing proteins did not copurify in significant quantities (data not
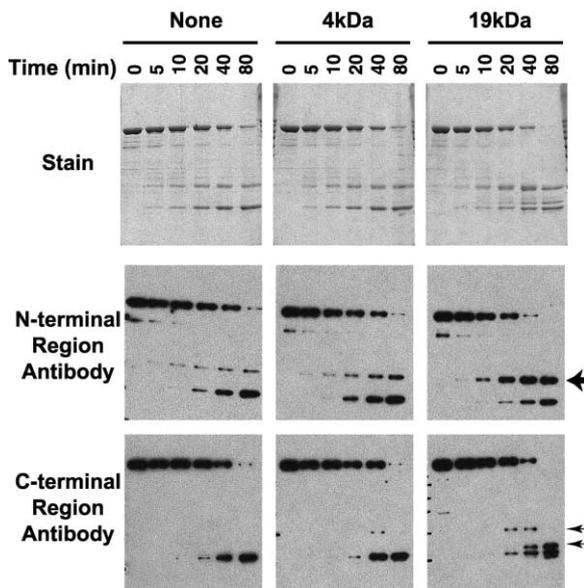
Figure 4. pK^L-Induced Conformational Change in Hsp104 Identified by V8 Proteolysis

Coomassie-stained gels of proteolytic digests are shown in the top panel. The appearance of the N-terminal and the C-terminal fragments was followed by immunoblotting with Ab (2-3) (middle panel) or Ab (8-1) (lower panel), respectively. See Results for description.

shown). Each mutant yielded a clear tryptophan fluorescence signal. None changed in the presence of 4 kDa pK^L; one, Y507W, changed in the presence of 19 kDa pK^L, (Figure 3B). Specifically, pK^L caused a 6–7 nm blue shift in the wavelength for maximum fluorescence, indicating that tryptophan 507, in the middle region of Hsp104, becomes partially buried in response to pK^L binding. A similar shift, albeit of lesser magnitude, was observed with histone H1 (data not shown). Thus, the binding of pK^L and histone H1 to the CTD appears to induce a conformational change in the middle region of Hsp104.

PK^L-Induced Conformational Change in the Middle Region Revealed by Proteolytic Sensitivity
To probe more globally for pK^L-induced conformational changes, we subjected Hsp104 to proteolytic digestion. Endoproteinase Glu-C (V8 protease) cleaves after negatively charged amino acids and thus avoids the complication of pK^L cleavage during the reaction (histone H1 could not be used in this assay since it is readily cleaved by V8). V8 provides an excellent probe for conformational changes in Hsp104 because, in the absence of structural constraints, there are 150 possible cleavage sites.

Hsp104 digestion patterns were not affected by 4 kDa pK^L, but with 19 kDa pK^L, the protein became more sensitive to digestion, and the distribution of sensitive sites changed (Figure 4). To facilitate comparison, protease concentrations were adjusted in these experiments to yield similar digestion time courses. All changes were detected by Coomassie staining and by immunoblotting with region-specific antibodies mapped to the middle region.

In the absence of pK^L, the major proteolytic product reacting with an antibody specific for the N-terminal NBD results from cleavage immediately after residue 360, which excludes the middle region (Hattendorf and Lindquist, 2002b). A minor ~48 kDa fragment (thick arrow, Figure 4) that must include a segment of the middle region becomes much more prominent in the presence of 19 kDa pK^L. In the absence of pK^L, the major fragment reacting with an antibody specific for the C terminus results from cleavage immediately after amino acid 548 (Hattendorf and Lindquist, 2002b). After pK^L binding, two larger fragments become prominent (thin arrows, Figure 4). Since all contain the extreme C terminus of Hsp104, the larger fragments must represent cleavages N-terminal to residue 548, that is, in the middle region.

Effect of Monoclonal Antibodies on the ATPase Activity of Hsp104
To provide another set of tools for defining regions critical in regulating Hsp104's activities, we generated a panel of monoclonal antibodies. Mice were immunized with full-length Hsp104, and hybridoma clones secreting Hsp104-specific antibodies were rescreened for recognition of different epitopes with immunoblots derived from the digestion of Hsp104 with pepsin, papain, or trypsin. Clones that yielded different patterns on these blots were selected and their regional specificities determined by immunoblotting against cloned fragments of Hsp104 (Figure 5A).

Next, we asked how each antibody affected the basal and pK^L-stimulated ATPase activity of Hsp104 (Figure 5B). NBD1 contributes the majority of the basal ATPase activity of Hsp104 (Parsell et al., 1994a; Schirmer et al., 1998, 2001). Antibody 46E, which recognizes NBD1 (Figure 5A), reduced basal activity by just 3-fold (Figure 5B). Strikingly, 46E eliminated pK^L-stimulated ATP hydrolysis. Similar results were obtained when only the F$_{ab}$ fragment of this antibody was tested (data not shown). These observations, together with analysis of NBD mutations (see below), indicate that in addition to contributing to the basal ATPase activity of Hsp104, NBD1 makes a strong contribution to the stimulated hydrolysis that occurs when pK^L binds the CTD.

Antibodies 14B, 28B, and 3D3, which recognize the poorly understood region N-terminal to NBD1, had little effect on basal- or pK^L-stimulated ATPase activity of Hsp104 (Figure 5B). Either this domain makes little contribution to pK^L-stimulated ATPase activity or these antibodies do not interact with the domain in an informative manner. Antibody 27B, which binds to the CTD of Hsp104, blocked pK^L-stimulated ATPase activity, providing independent confirmation of the C-terminal region's role in this activity (Figure 5B).

All three antibodies that mapped to the middle region (4G10, 4B, and 17B) stimulated the basal ATPase activity of Hsp104 to levels comparable to those achieved with pK^L. Further, in the presence of these antibodies, the ATPase activity could not be additionally stimulated by pK^L (Figure 5B). Thus, the binding of antibodies to the middle region of Hsp104 bypasses the effect of pK^L binding at the CTD. This suggests that the conformational changes in the middle region are not just an insubstantial consequence of pK^L binding, but part of the

A

B

C
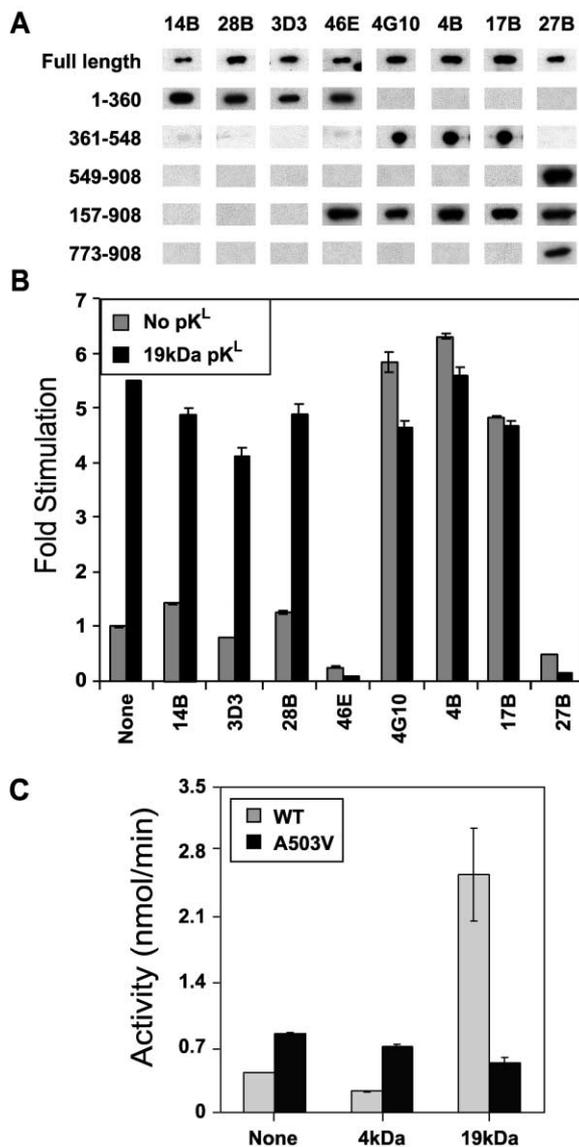
Figure 5. Role of the Middle Region of Hsp104 in the Stimulation of ATP Hydrolysis

(A) Immunoblots of the different monoclonal antibodies (arranged in the order of their epitopes in Hsp104 sequence) against various fragments of Hsp104 (shown left).
(B) Effect of monoclonal antibodies on the ATPase activity of Hsp104 in the absence (gray bars) and presence of 19 kDa pK^L (black bars). The ATPase activities were normalized to that in the absence of monoclonal antibodies or pK^L.
(C) ATPase activities of wild-type Hsp104 (light gray bars) and the middle region mutant, A503V (dark gray bars), in the absence (None) and presence of 4 kDa or 19 kDa pK^L.

mechanism that drives polypeptide stimulated ATP hydrolysis.

Effects of Mutations in the Middle Region

An entirely different approach to identifying regions critical for regulating the Hsp104 reaction cycle is to screen for mutations that affect its function. In a separate genetic study to identify regions required to regulate Hsp104's protein remodeling activity, all of the mutants

carried at least one mutation in the middle region of Hsp104 (E.C.S., O.R. Homann, and S.L.L., unpublished data). Here, to determine how such mutations might affect basal and pK^L-stimulated ATPase activity, we examined the simplest mutation, a single amino acid substitution in the middle region, alanine to valine at position 503.

The A503V mutant exhibited higher levels of basal ATPase activity than wild-type Hsp104, though not as high as wild-type protein bound to pK^L (Figure 5C). More strikingly, the ATPase activity of the A503V mutant was not further stimulated by the addition of pK^L (Figure 5C). These changes did not reflect defects in pK^L binding; the mutant protein bound pK^L-coupled Sepharose beads just as well as the wild-type protein did (data not shown). Thus, a single amino acid substitution in the middle region completely disrupted the normal communication of signals from the CTD to the NBDs of Hsp104. Most importantly, it did not impair the ATPase activity of Hsp104. Rather, it kept the protein in a partially activated state, whether pK^L was bound or not. This confirms that the middle region plays a central role in driving the ATP hydrolysis that occurs in response to peptide binding at the C terminus.

Effects of Mutations in the Two NBDs

Next, we dissected the roles of the two NBDs. We first tested a double-point mutant in NBD1 (K218T:A315T) that carries a threonine substitution in the critical lysine in the Walker A site and an alanine to threonine substitution in the region of sensor 1 (see below). This protein has a CD spectrum indistinguishable from that of wild-type Hsp104 and assembles into hexamers as efficiently. However, its basal rate of ATP hydrolysis is only ~2% that of wild-type protein, reflecting the small contribution that NBD2 makes to basal ATPase activity (Schirmer et al., 1998, 2001; Hattendorf and Lindquist, 2002a). With the addition of 19 kDa pK^L (Figure 6A), ATPase activity increased several fold, but remained much lower that that of the wild-type protein. Apparently, NBD2 responds to the binding of polypeptide at the CTD with increased hydrolysis, but (confirming the conclusion reached with antibody 46E) NBD1 is required for the full response to peptide binding.

To test the role of NBD2 in the stimulation of NBD1, we made use of a new mutant that specifically inactivates NBD2 without compromising hexamer assembly (Schirmer et al., 2001; Hattendorf and Lindquist, 2002a). AAA proteins contain a conserved polar residue, known as sensor 1, which is in the vicinity of the γ-phosphate of ATP (Neuwald et al., 1999). The sensor 1 residue of Hsp104's NBD2 has recently been identified as the asparagine at position 728, and mutating this residue to alanine (N728A) eliminates Hsp104 function in thermotolerance and prion maintenance. This mutation had no effect on hexamer assembly or nucleotide binding affinity but effectively eliminates ATP hydrolysis at NBD2 (Hattendorf and Lindquist, 2002a). This mutant binds to pK^L as well as the wild-type Hsp104 (data not shown). Thus, N728A provides an ideal tool to assay the importance of ATP hydrolysis at NBD2 in communicating signals from the CTD to NBD1. The ATPase activity of N728A was stimulated by pK^L only 1.3-fold, compared
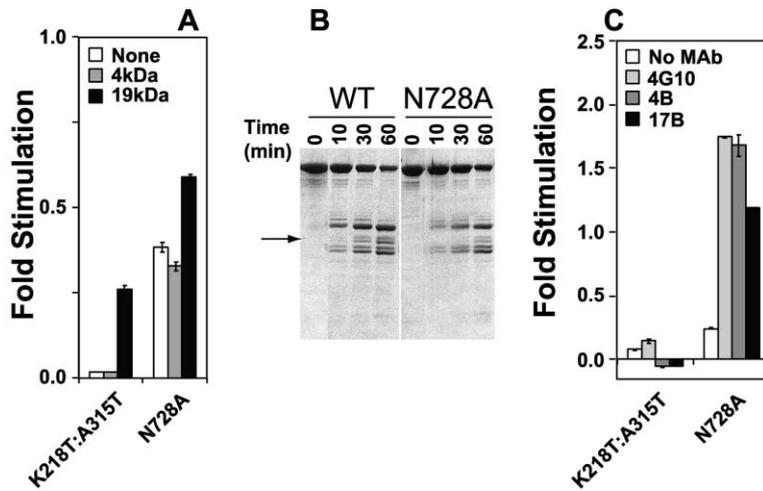
Figure 6. Roles of the Two Nucleotide Binding Domains in ATPase Stimulation

(A) Effect of pK^L on the ATPase activities of K218T:A315T and N728A mutants of Hsp104. ATPase activity was measured in the absence (white bars) or in the presence of 4 kDa (light gray bars) or 19 kDa (dark gray bars) pK^L.

(B) Coomassie-stained SDS-PAGE of V8 proteolysis of wild-type (left) and N728A (right) in the presence of 19 kDa pK^L. The pK^L-induced conformational change, as measured by an increased sensitivity to proteolysis in the middle region (fragments marked by arrow), was greatly reduced in the N728A mutant.

(C) Effect of stimulatory middle region antibodies 4G10 (light gray bars), 4B (dark gray bars), and 17B (black bars) on the ATPase activity of the two mutants. The fold stimulations in (A) and (C) are normalized for the ATPase activity of wild-type Hsp104 in the absence of pK^L or monoclonal antibodies.

with the ∼6–8 fold stimulation observed with wild-type Hsp104 (Figure 6A). Thus, the ATPase cycle of NBD2 plays an important role in communicating peptide binding at the CTD to NBD1.

Since conformational change in the middle region is also involved in communicating peptide binding at the CTD to NBD1, we asked if ATP hydrolysis at NBD2 helps to drive this change in the middle region. To do so, we examined the alteration in proteolytic digestion that occurs in the middle region when the CTD binds pK^L. The alteration was much less pronounced in the NBD2 mutant N728A than in the wild-type protein (Figure 6B).

To determine if the middle region can itself drive hydrolysis in NBD1, we tested the effects of the monoclonal antibodies 4G10, 4B, and 17B. None of the antibodies stimulated hydrolysis in protein containing the NBD1 mutation, K218T:A315T. All three strongly stimulated hydrolysis in proteins containing the NBD2 mutation, N728A (Figure 6C). The ability of the middle region antibodies to bypass the requirement for hydrolysis at NBD2 confirms the importance of the middle region in driving hydrolysis at NBD1 (just as they can function to bypass the requirement for polypeptide binding at the CTD).

Discussion

The AAA module has been broadly utilized in nature in combination with other domains to achieve highly specialized protein remodeling functions in an extraordinary variety of systems. However, the manner in which ATPase activity is coupled to substrate occupancy to produce conformational changes has yet to be defined for any of these proteins. We report an interdomain signal transduction pathway in Hsp104, one of the larger members of the family, with two AAA modules per monomer: peptide binding at the CTD alters the ATPase cycle at NBD2, which induces conformational change in the middle region that, in turn, drives hydrolysis at NBD1 (Figure 7).

Deciphering this pathway depended upon applying a variety of techniques that could only have been employed with a tractable peptide that forms stable com-

plexes with Hsp104. The K$_d$ for pK^L binding (inferred by ATP hydrolysis) is in the nanomolar range and the off-rate is low enough to retain Hsp104 on pK^L-coupled beads through several rounds of washing. Previous attempts to perform such analyses with natural Hsp104 substrates have been thwarted by the nature of these substrates (aggregates), the transience of their interactions with Hsp104, and the fact that these interactions are not stabilized by other nucleotides or analogs. Certainly, Hsp104 can act on substrates that are not lysine rich (Parsell et al., 1994b; Glover and Lindquist, 1998), but several lines of evidence suggest that lysine-rich regions provide one important component of normal substrate recognition. The first identified Hsp104 substrates were the aggregates that form during heat shock, most prominently in the nucleus (which contains many lysine-rich proteins). To identify the heat shock substrates of Hsp104, we analyzed proteins that were not resolubilized after a mild heat shock in Hsp104-deficient cells but were resolubilized in wild-type cells. Even when considering proteins with pIs between 4 and 7, which should be biased against basic amino acids, the identified proteins contained lysine-rich subsequences, e.g.,
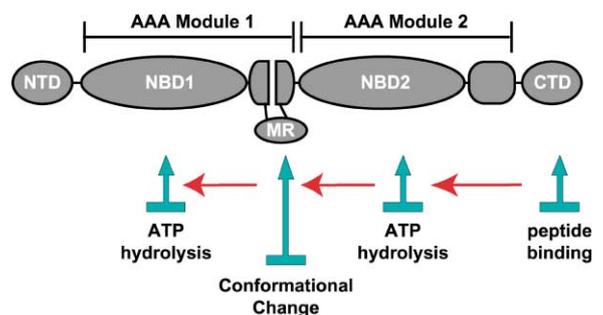
Figure 7. Pathway of Communication between the Domains of Hsp104

Peptide binding at the C-terminal domain (CTD) influences the ATPase cycle of the C-terminal NBD (NBD2), which in turn causes a conformational change in the middle region (MR) that results in the stimulation of the N-terminal NBD (NBD1). We do note that this pathway need not essentially be unidirectional.

Yef3, Rrp46, and ykl056c (A.G.C. and S.L.L., unpublished data). The best known substrate for Hsp104 in the absence of stress is the NM region of Sup35 (Li and Lindquist, 2000). A single 90 amino acid segment in this region contains 23 lysines and no arginines. Replacing this region with other charged amino acid segments creates Sup35 variants that are still capable of existing in either the soluble nonprion form or the insoluble prion form. However, prion propagation with these variants has lost its strong sensitivity to Hsp104 (J.J. Liu, N.J. Sondheimer, and S.L.L., unpublished data). We cannot exclude the possibility that pK^L may mimic some as yet unknown effector protein. It seems more likely however, that the 19 kDa pK^L mimics aggregated proteins with conformationally flexible, polar (charged) surfaces that bind Hsp104 but cannot complete the normal reaction cycle. In either case, the biological relevance of its interaction with Hsp104 is clear: (1) pK^L-induced stimulation of Hsp104 ATPase activity could be blocked in a conformationally specific manner by the prion domain of Sup35; and (2) 19 kDa pK^L, which binds Hsp104 cooperatively and with high affinity, blocks Hsp104-mediated reactivation of aggregated firefly luciferase much more effectively than 10 kDa pK^L, which binds noncooperatively with low affinity. Thus, 19 kDa pK^L acts much like a mutation that provides a tool for deciphering a complex pathway by blocking it in action.

With this tool in hand, other methods allowed us not only to elucidate the importance of three other domains in the response of Hsp104 to polypeptide binding but also to define the order in which they function and determine what drives each step. For example, the function of NBD2 (not just nucleotide binding, but hydrolysis as well) is required to stimulate hydrolysis at NBD1. Moreover it is the conformational change in the middle region that drives communication between the two NBDs. Mutations in the middle region uncouple the two NBDs, and antibody binding to the middle region of the wild-type protein drives NBD1 hydrolysis even when the CTD has not engaged polypeptide and NBD2 has not hydrolyzed nucleotide. In separate work, we have found that NBD1 is responsible for most ATP hydrolysis in Hsp104 (Schirmer et al., 1998; Hattendorf and Lindquist, 2002a), but NBD2 binds ATP with much greater affinity than NBD1, consistent with the regulatory role we have detected for it here. Interestingly, in ClpA, another protein with two AAA modules, the properties of the two NBDs are apparently reversed. Such differences, together with the nature of binding specificities in the highly divergent N and C termini may determine directionality of substrate translocation and effector molecule interactions, within a general framework of sequential polypeptide binding, ATP hydrolysis, conformational change, and again ATP hydrolysis.

The middle region is the most variable in size and sequence among the Hsp100/Clp family and hence was previously called the "spacer" region. In Hsp104, the middle region contains a segment with very high propensity to form a dimeric coiled coil (score of 0.93 with the MULTICOIL program, the fourth highest scoring polypeptide in the entire yeast proteome; Wolf et al., 1997; Newman et al., 2000). The isolated peptide does not form coiled coils on its own (M.S.R. and S.L.L., unpublished data). However, such a structure is very likely relevant

to function; all members involved in stress tolerance have a middle region with a high propensity to form coiled-coil structure (A.G.C., unpublished data) despite high sequence divergence (Schirmer et al., 1996). Clearly, the middle region plays a vital role in the response of Hsp104 to polypeptide binding. The conformational change we observe in the middle region (in the context of the whole protein, perhaps substrate and ATP-regulated breakage and formation of a coiled coil) may act as a hinge between substrate binding sites to propel large segments of polypeptide in an unfolding reaction geared toward the disaggregation of stress-damaged proteins and the remodeling of prion substrates.

The inherent conformational flexibility required for such a mechanism might explain why efforts to crystallize Hsp104 have been unsuccessful to date. We have described a set of tools (mutations, substrates, and antibodies) that stabilize Hsp104 at several different points in its reaction cycle to facilitate and enhance future structural studies. Here, they have provided a framework for understanding the remarkable interdependent, dynamic nature of Hsp104 domain interactions.

Experimental Procedures

Materials
Test substrates were from Sigma Chemical Co. (St. Louis, MO). ATP and Endoproteinase Glu-C from Roche Molecular Biochemicals, (Mannheim, Germany) and NHS-activated Sepharose 4 Fast flow from Amersham Pharmacia Biotech (Piscataway, NJ). The NM region of Sup35 was purified as described (Glover et al., 1997).

Cloning and Protein Purification
Wild-type Hsp104 was purified from *S. cerevisiae* as described (Schirmer and Lindquist, 1998), with a Sephacryl S-400 (Amersham Pharmacia Biotech) column in place of the final DEAE column. Hsp104 mutants A503V, K218T:A315T, and N728A, were purified from RIL-Codon-plus *E. coli* cells (Stratagene, La Jolla, CA) as described (Hattendorf and Lindquist, 2002a).

The tryptophan substitutions were made by polymerase chain reaction (PCR) mutagensis in pBluescript SK—mutagenic primers were as follows: F130W, CTACTTGAGCTTCCTTCCATATTTGCTG AATAGAC; Y257W, CTTCGAAATCACCTTTCCATTTAGCACCTGCG; Y466W, CTCTAAGACAACGTTGGAATGAAGAAAAGCATG; Y507W, CCGCTGATTTAAGGTGGTTCGCCATCCCAG; L553W, CAGGGATA CCAGTCCATCTTGCAGCTGTTTC; Y819W, CCCATATCATCGGAC CAACCATATTTGGCCA; and Y892W, CATTATCGTCATCACCCCAC GTGTCAGCCCC. The mutagenized fragments were sequenced and placed into the full-length HSP104 gene in pJC45 for expression in *E. coli* BL21 (DE3) (Clos and Brandau, 1994) or in pGALSc104b for expression in *S. cerevisiae* (Schirmer et al., 1994). 10× histidine-tagged mutant proteins were purified as described (Schirmer and Lindquist, 1998).

The Hsp104 fragments, residues 1–575 and 773–908 were cloned by PCR (1–575 forward, GGAAAACAAGGCCCTTGATG; 1–575 reverse, GAGCTCTTAGTCACGTTCCATATG; 773–908 forward, TTT TTGGATCCGGGATGAACAAGCTATCTAGAAAAGCTAT; and 773–908 reverse, GGTAACGCCAGGGTTTTCC) into pPRO-EX-HTb (Life Technologies, Rockville, MD) in addition to fragments 1–360, 1–548, and 549--908 (D.A.H. and S.L.L., unpublished data). The pPROEX-HTb-Hsp104 (157–908) was a kind gift from Dr. Sukyeong Lee. All fragments were expressed in RIL-Codon-plus cells and purified as follows: protein in the soluble fraction was purified on a Ni-NTA column (Qiagen, Valencia, CA); the histidine tag was removed using the TEV protease (Life Technologies); and the protein was further purified over a DEAE-Sepharose (Amersham Pharmacia Biotech) column.

The middle region of Hsp104 (residues 361–548) was cloned into

pJC45 by PCR (forward primer, AAAAGGATCCGGGATGATACATCA
TGGT; reverse primer, AAAAGAGCTCCTATTCAGAAATGGTGTC).
Induced *E. coli* lysate was used for immunoblotting in Figure 5A.

Monoclonal Antibodies
Mice were immunized with Hsp104 in Freund's complete adjuvant
and boosted with incomplete adjuvant. The dispersed spleen cells
from the immunized mouse were fused to sp2/0 myeloma cells using
polyethylene glycol (PEG) and selected in HAT medium. Hybridoma
fusion clones were first screened with whole Hsp104 by enzyme-
linked immunosorbant assays (ELISA). Positive clones were re-
screened for different specificities by reacting culture supernatants
with immunoblots containing Hsp104 digested with three different
proteases: pepsin, papain, and trypsin. Positive clones were ampli-
fied and stabilized by repeated rounds of serial dilution.

ATPase Activity
ATPase activity of Hsp104 was measured as described (Schirmer
et al., 1998) either in $1\times$ HPS (10 mM HEPES [pH 7.5], 140 mM KCl,
15 mM NaCl, and 10 mM MgCl$_2$; Figures 1, 2A, and 2B) or in $1\times$
RFB (20 mM HEPES [pH 7.5], 50 mM KCl, and 10 mM MgCl$_2$; all
other Figures). The fold stimulation with pK^L is higher in $1\times$ HPS
due to lower basal ATPase activity. Assays contained ATP at 5 mM,
Hsp104 at 0.2 μM, and polypeptides at 1 μM. Activities with different
polypeptides were normalized to that in the absence of polypep-
tides.

ATPase activity of Hsp104 with different concentrations of pK^L
was analyzed using GraFit version 4.00 software (Erithacus Software
Limited, Middlesex, UK). The data were fitted to the ligand-binding
equation,

$$A = ([L]^n \times A_{max})/(K_d^n + [L]^n) + B,$$

where A is activity at a given pK^L concentration [L]; A$_{max}$ is the
maximally stimulated activity; K$_d$ is the binding constant; n is the
Hill coefficient; and B is the activity in the absence of pK^L.

Effects of antibodies on ATPase activity were tested after incubat-
ing Hsp104 with each monoclonal antibody in $1\times$ RFB without ATP
for 2 hr on ice. ATPase activity was measured as described above.

Bead Binding Assays
To prevent multiple cross links per pK^L molecule, the number of
cross-linkable sites was reduced by pretreating NHS-activated
Sepharose beads with 10 mM ethanolamine as a 50% (v/v) suspen-
sion on ice for 1 hr. Beads were cross-linked either to mixtures of
ethanolamine and 4 kDa pK^L (at 0, 0.1, 0.2, 0.5, 1, 2, 5, or 10 mM)
or to 0.1mM of 19 kDa pK^L containing 5 mM ethanolamine, in $1\times$
RFB for 2h at 25°C. The remaining NHS sites were blocked by incu-
bating the beads in 1 M ethanolamine for 1 hr at 25°C. The cross-
linked beads were washed as per manufacturer's suggestions and
stored in $1\times$ RFB at 4°C.

To test the effect of localized crowding of 4 kDa pK^L, Hsp104 was
added to beads cross-linked at different densities. To identify the
pK^L binding domain, purified Hsp104 fragments were added to vari-
ous ratios of beads cross-linked to 19 kDa pK^L and mock-treated
beads. The amount of protein remaining in the supernatant was
analyzed by SDS-PAGE.

Analysis of Tryptophan Mutants
The ability of tryptophan substituted Hsp104 mutants to confer ther-
motolerance was tested as described (Schirmer et al., 1994).

Fluorescence spectra between 305 and 400 nm of all seven trypto-
phan mutants were measured with or without pK^{L}s of different chain
lengths in $1\times$ RFB containing 5mM ATP by excitation at 295 nm
with 5 nm bandpass using a Jasco FP750 spectrofluorometer.

Proteolysis
To identify conformational changes in Hsp104 associated with pK^L
binding, V8 proteolysis was carried out in $1\times$ RFB containing 5 mM
ATP at 37°C with or without 1 μM 4 kDa or 19 kDa pK^{L}s. Aliquots
were taken out at specified times and proteolysis was stopped by
the addition of AEBSF to 5 mM. Samples were subjected to SDS-
PAGE followed by immunoblotting with N-terminal-specific (2-3) or
C-terminal-specific (8-1) antibodies.

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