Towards a Mechanistic Understanding of the Molecular Chaperone Hsp104

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

The AAA⁺ chaperone Hsp104 mediates the reactivation of aggregated proteins in Saccharomyces cerevisiae and is crucial for cell survival after exposure to stress. Protein disaggregation depends on cooperation between Hsp104 and a cognate Hsp70 chaperone system. Hsp104 forms a hexameric ring with a narrow axial channel penetrating the centre of the complex. In Chapter 2, I show that conserved loops in each AAA⁺ module that line this channel are required for disaggregation and that the position of these loops is likely determined by the nucleotide bound state of Hsp104. This evidence supports a common protein remodeling mechanism among Hsp100 members in which proteins are unfolded and threaded along the axial channel. In Chapter 3, I use a peptide-based substrate mimic to reveal other novel features of Hsp104’s disaggregation mechanism. An Hsp104-binding peptide selected from solid phase arrays recapitulated several properties of an authentic Hsp104 substrate. Inactivation of the pore loops in either AAA⁺ module prevented stable peptide or protein binding. However, when the loop in the first AAA⁺ was inactivated, stimulation of ATPase turnover in the second AAA⁺ module of this mutant was abolished. Drawing on these data, I propose a detailed mechanistic
model of protein unfolding by Hsp104 in which an initial unstable interaction involving the loop in the first AAA$^+$ module simultaneously promotes penetration of the substrate into the second axial channel binding site and activates ATP turnover in the second AAA$^+$ module. In Chapter 4, I explore the recognition elements within a model Hsp104-binding peptide that are required for rapid binding to Hsp104. Removal of bulky hydrophobic residues and lysines abrogated the ability of this peptide to function as a peptide-based substrate mimetic for Hsp104. Furthermore, rapid binding of a model unfolded protein to Hsp104 required an intact N-terminal domain and ATP binding at the first AAA$^+$ module. Taken together, I have defined numerous structural features within Hsp104 and its model substrates that are crucial for substrate binding and processing by Hsp104. This work provides a theoretical framework that will encourage research in other protein remodeling AAA$^+$ ATPases.
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List of Abbreviations

AAA+  ATPases associated with a variety of cellular diseases
ADP  Adenosine diphosphate,
AMPPNP  Adenylyl-imidodiphosphate
ATP  Adenosine triphosphate,
ATPγS  Adenosine-5’-O-(3-thiotriphosphate)
CCD  coiled-coil domain
Clp  caseinolytic protease
cryo-EM  cryoelectron microscopy
CTE  C-terminal extension
D1  first AAA⁺ module
D2  second AAA⁺ module
∆F  change in fluorescence intensity
DUR  delayed upregulation
EDC  N-ethyl-N’-(dimethyl-aminopropyl)-carbodiimide
FFL  firefly luciferase
FITC  fluorescein isothiocyanate
fRCMLa  fluorescein-labeled reduced carboxymethylated \( \alpha \)-lactalbumin
FRET  fluorescence resonance energy transfer
GFP  green fluorescent protein
HOP  Hsp-organizing protein
HSE  heat shock element
Hsp  heat shock protein
Kd  equilibrium dissociation constant
Km  Michaelis-Menten constant
koff  dissociation rate constant
kon  association rate constant
NAC  nascent chain-associated complex
NADH  Nicotinamide adenine dinucleotide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NBD</td>
<td>nucleotide binding domain</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
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<td>NTD</td>
<td>N-terminal domain</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RAC</td>
<td>ribosome-associated complex</td>
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<td>RCMLa</td>
<td>reduced carboxymethylated α-lactalbumin</td>
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<tr>
<td>SBD</td>
<td>substrate binding domain</td>
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<tr>
<td>SD</td>
<td>small domain (of AAA⁺ module)</td>
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<tr>
<td>sHsp</td>
<td>small heat shock protein</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>STRE</td>
<td>stress response element</td>
</tr>
<tr>
<td>sulfo-SMCC</td>
<td>sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>TF</td>
<td>trigger factor</td>
</tr>
<tr>
<td>TPR</td>
<td>tetratricopeptide-repeat</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum rate of enzyme activity</td>
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1 Introduction

The focus of this thesis is a unique cellular response mechanism to protein misfolding and aggregation in *Saccharomyces cerevisiae* – the unfolding and extraction of polypeptides from a protein aggregate by the molecular chaperone Hsp104. In Chapter 2, I discuss the role of the axial channel in the resolubilization of protein aggregates by Hsp104. In Chapter 3, I identify a high affinity peptide for Hsp104 that was then used as a substrate mimetic to probe biochemical aspects of Hsp104’s protein remodeling function. In Chapter 4, I identify elements within a model Hsp104 binding peptide as well as structural elements in Hsp104 that are required for efficient substrate binding. Finally, in Chapter 5, I discuss the remaining questions regarding the protein remodeling mechanism of Hsp104.

I begin this Introduction with a brief discussion of how proteins fold into their native conformations and the role of molecular chaperones in facilitating this process, a more detailed treatise on the causes of protein misfolding and the two main cellular responses employed to deal these stresses – the expression of molecular chaperones, the upregulation of the chemical chaperone trehalose, and the synergy between these two unique pathways - will follow. Finally, I present a detailed introduction on the structural and functional details of the yeast protein disaggregate Hsp104.

1.1 Protein folding in the cell and the role of molecular chaperones

A central process in biology is the conversion of genetic information into the functional proteins that carry out the genetic programme. Ribosomes have an essential
role in this process as they translate mRNA into linear polypeptides. There is, however, a further step — the folding of the newly translated polypeptides into well-defined, three-dimensional conformations. Proteins known as molecular chaperones are crucial for this final step in the readout of genetic information.

1.1.1 The protein folding landscape

Newly synthesized polypeptides emerge from the ribosome in an unfolded state. In recent years, a number of proteins have been shown to fold spontaneously \textit{in vitro} (Jackson 1998), supporting Anfinsen’s initial postulation that the primary sequence of a protein contains all the information required to specify a precise three-dimensional structure for biological activity (Anfinsen 1973). It is generally accepted that proteins greater than 100 amino acids in size, which constitute greater than 90% of all proteins, fold via one or more intermediate states that act as stepping stones to the native state (Brockwell and Radford 2007). The conformational space accessible to a folding multi-domain protein is even larger and yet these proteins fold to their native state on a biologically relevant timescale (Kubelka \textit{et al.} 2004). In a landscape view of protein folding, the denatured protein samples a large ensemble of structures and multiple folding pathways with an inevitable goal to lower its free energy and increase its stability as native contacts form. Non-native contacts also appear to be a common feature of folding energy landscapes, playing a role in the folding of proteins spanning all structural classes. As long as kinetic traps do not develop, the formation of non-native contacts might increase the rate of folding by introducing bias into the folding pathway (McLeish 2005).
1.1.2 Protein folding *in vivo*

In contrast to protein folding *in vitro*, protein folding in live cells occurs co-translationally as the polypeptide emerges from the ribosome. This may occur even before a complete domain has been synthesized and therefore initiates the formation of a partially folded intermediate as the unfolded polypeptide collapses to a thermodynamically-stable state. These intermediates therefore often represent major kinetic traps in the folding pathway of many proteins. The commonality of intermediates during folding also rationalizes the need for a set of proteins called molecular chaperones that are evolutionarily conserved in all forms of life, so as to avoid problems arising from potential aggregation of these partially folded proteins.

*In vivo*, aggregation of folding intermediates is exacerbated by an extremely crowded environment (approximately 300 mg/mL) with high concentrations of proteins, nucleic acids, and other macromolecules. This crowding effect strongly enhances the formation of intermolecular interactions. For a folding polypeptide, crowding can exaggerate non-native interactions with other unfolded/partially folded proteins mediated by exposed hydrophobic residues and ultimately the formation of toxic off-pathway aggregates (Ellis and Minton 2006).

1.2 Molecular chaperones and protein folding *in vivo*

Molecular chaperones can be broadly defined as any protein that interacts, stabilizes, or helps a non-native protein achieve its native conformation but is not present in the final functional structure. Chaperones are involved in a wide range of cellular functions including regulated protein expression and degradation, oligomeric assembly,
and intracellular transport. Two functions of chaperones that are most relevant to this thesis are de novo protein folding and refolding of stress-denatured proteins. Molecular chaperones recognize hydrophobic side-chains of polypeptide segments in non-natively folded proteins and promote proper folding through cycles of binding and release. High affinity chaperone binding to exposed hydrophobic segments effectively prevents the formation of stable, irreversible protein aggregates. Cycles of binding and release of the unfolded protein segment provide renewed opportunities for the protein to fold to the native state. In this way, chaperones do not act by contributing steric information but instead by increasing the efficiency of folding. A number of essential proteins have extremely poor intrinsic folding efficiencies and essentially cannot fold properly without the aid of molecular chaperones (Gong et al. 2009). Actins and tubulins have energetically unfavourable folding pathways and can overcome kinetic barriers to folding only through the assistance of chaperones (Neirynck et al. 2006; Yam et al. 2008).

Numerous classes of molecular chaperones have been described to date, many of which are known as heat shock proteins or stress proteins since they are upregulated when cells are exposed to stresses that increase the concentration of aggregation-prone folding intermediates (reviewed in (Young et al. 2004). Because of the cytotoxicity of protein aggregates, molecular chaperones are central components of the cellular machinery against protein-misfolding diseases and aging.

Substrate binding and release by chaperones is regulated using distinct mechanisms among different chaperone families. ATP binding and hydrolysis can regulate the cycles of substrate binding and release in many but not all chaperones. Once bound by chaperones, partially folded proteins are effectively prevented from forming
non-native interactions, reducing the concentration of folding intermediates and
decreasing the risk of misfolding and aggregation. For some small heat shock proteins, a
transition to the high-affinity substrate binding state requires a temperature-dependent
conformational change that reveals a hydrophobic substrate binding site specifically at
elevated temperatures (Nakamoto and Vigh 2007). In contrast with high-temperature
induced substrate binding, phosphorylation lowers both in vitro and in vivo chaperone
activity in other small heat shock proteins (Nakamoto and Vigh 2007).

Most chaperones can be generally grouped into five highly conserved families:
the non-ATPase small heat shock proteins (sHsps), and the ATPases Hsp60, Hsp90,
Hsp70, and Hsp100. sHsps bind to misfolded proteins and form a network that
accumulates and sequesters aggregation-prone polypeptides into supermolecular
structures that are then either refolded or targeted for degradation (Nakamoto and Vigh
2007). Hsp60s, otherwise known as the chaperonins GroEL/ES in prokaryotes and
Tric/CCT in eukaryotes, form an oligomeric cavity that sequesters an unfolded
polypeptide from the cellular milieu and allows the protein to fold in the absence of
unproductive intermolecular interactions (Horwich et al. 2006). Hsp90s are dimeric
chaperones that function downstream of more traditional folding chaperones and regulate
the final stages of folding and consequently the temporal activity of a large array of client
proteins that includes many signaling molecules (Pratt et al. 2008). A brief treatise on
the Hsp70 and its J-domain containing Hsp40 cochaperone families, and a detailed
description of the Hsp100 protein remodeling chaperones follows.
1.2.1 Hsp70/J protein reaction cycle

The Hsp70 family is perhaps the most widely studied molecular chaperone family because it plays essential roles in protein biogenesis, transport, as well as degradation, and is involved in a number of human pathologies ranging from cancer to protein aggregation diseases.

All Hsp70 molecules have the same conserved architecture consisting of an amino-terminal adenine nucleotide binding and hydrolysis domain (NBD) of roughly 45 kDa and a carboxy-terminal substrate-binding domain (SBD) of approximately 27 kDa (Morano 2007). The SBD can be subdivided further into a substrate-binding pocket consisting of two β-sheets and “lid,” composed of a pair of α-helices, whose conformation controls the affinity of the SBD (Fig 1.1A). Hsp70 proteins recognize short hydrophobic polypeptide stretches that are in an extended conformation (Flynn et al. 1991; Blond-Elguindi et al. 1993; Gragerov and Gottesman 1994; Rudiger et al. 1997) and exhibit two stable substrate-binding conformations that are controlled by the nucleotide occupancy of the NBD (Fig 1.1B). ATP-bound Hsp70 has a SBD that binds substrates with low affinity due to a mobile lid domain that fails to effectively “clamp” substrates and therefore exchanges polypeptide substrates rapidly. In the ADP-bound state, the lid domain is lowered onto the substrate effectively locking the substrate into a tight association with the SBD (Flynn et al. 1989; Palleros et al. 1991). Iterative cycles of nucleotide binding, hydrolysis, and release control these two states and allow the Hsp70 to assist in the productive folding of a substrate protein. However, optimal polypeptide folding might require the coordinated release of the polypeptide from several Hsp70 monomers, although, as yet, there is no evidence for such coordination. An
extensive array of co-chaperones interacts with and modulates the ATPase activity and binding specificities of Hsp70s. The co-chaperone Hsp40 can bind substrate polypeptides itself, and its J domain, a highly conserved helical domain, binds to and activates ATP hydrolysis by Hsp70, which results in the transfer of polypeptides to Hsp70 (Li et al. 2009). Binding of folding intermediates to Hsp70 prevents protein aggregation while the subsequent release of the polypeptide substrate after ADP release effectively provides the substrate renewed opportunities to fold to the native state. In prokaryotes, nucleotide exchange is induced by the co-chaperone GrpE returning Hsp70 to the low-affinity ATP-bound state resulting in substrate release and completing the reaction cycle (Shaner and Morano 2007). Orthologues of GrpE are not found in the cytosol of eukaryotes, but nucleotide exchange by the 70-kDa heat-shock cognate protein (Hsc70) can be stimulated by the structurally unrelated mammalian co-chaperone BCL2-associated athanogene-1 (Bag1) and its homologues (Young et al. 2004). Mammalian HSP70-binding protein (HspBP1) and its *Saccharomyces cerevisiae* homologue Fes1 form another class of cytosolic Hsp70 nucleotide-exchange factors that are unrelated to GrpE (Young et al. 2004).

### 1.2.2 The ‘Bucket Brigade’ model

In the cytosol, a translating polypeptide encounters an array of molecular chaperones along its folding pathway. These chaperones can be functionally segregated into two classes: (i) chaperones that stabilize nascent polypeptides on ribosomes and initiate folding, and (ii) chaperones that act downstream in completing the folding process (Hartl and Hayer-Hartl 2009).
Figure 1.1 The Hsp70 chaperone. (A) Structural model of Hsp70. (i) Front and side views (left and centre) of the substrate binding domain (SBD) with a bound peptide (green) in a channel penetrating right through the bacterial Hsp70 (DnaK) SBD domain [pdb code 1dkx]. (ii) The ATPase domain of another member of the Hsp70 family, Hsc70 [pdb code 1kax]. The ATP (space filled) binding site is in a cleft. (B) The Hsp70/40 reaction cycle. An ATP-bound Hsp70 (i) interacts with a polypeptide (green line) that exposes a hydrophobic segment during biosynthesis or during cell stress (ii). Alternatively, a J-domain containing Hsp40 (purple) protein interacts with the misfolded polypeptide (iii) with a domain distinct from its Hsp70 binding site. The Hsp40-substrate complex subsequently interacts with Hsp70 in its ATP-bound form (iv). The substrate is passed from the J-domain of the Hsp40 to the SBD of Hsp70 (v). The J-domain interaction with Hsp70 stimulates hydrolysis of ATP resulting in a conformational change in the SBD that closes the ‘lid’ and prevents substrate release (high affinity state) and subsequently releasing the Hsp40. If needed, a nucleotide exchange factor (NEF) interacts with the ATPase domain of Hsp70 and stimulates exchange of ADP for ATP (vi). This in turn results in a conformational change in the SBD that permits the release of the bound substrate and returns Hsp70 to its initial low affinity state (i).
In prokaryotes, trigger factor (TF) is the first chaperone to meet nascent polypeptides as they emerge from the ribosome (Hoffmann and Bukau 2009). TF docks directly onto the ribosome specifically to the L23 and L29 ribosomal proteins, both of which are near the exit channel. This positions TF optimally to bind to long hydrophobic stretches in the elongating polypeptide to keep them soluble and prevent premature chain compaction until enough structural information is available for proper folding to occur (Hoffmann and Bukau 2009). TF also has a peptidyl-prolyl cis-trans isomerase activity although this activity is not critical for TF chaperone function. In both Archaea and Eukarya, the role of TF as the first general chaperone is functionally replaced by the nascent chain-associated complex (NAC) or the ribosome-associated complex (RAC), both of which are composed of an Hsp70 member and its associated Hsp40 homolog (Young et al. 2004).

Polypeptides that fail to fold after association with ribosome-bound chaperones, typically longer nascent chains that ultimately fold into multidomain structures, are co-translationally bound by members of the Hsp70 family (DnaK in bacteria, Hsc70 in eukaryotes). Co-translational domain folding is critical to avoiding non-native interdomain contacts, thus smoothing the energy landscape for large proteins. Hsp70s are also central organizers of the chaperone network and act to distribute subsets of proteins to other chaperones that act downstream, such as the Hsp60s and Hsp90s. The number of interacting substrates and the degree of functional redundancy among chaperones decreases from upstream (TF, Hsp70s) to downstream (Hsp60, Hsp90) chaperone components.
1.3 Protein aggregation during heat shock

A folded protein that maintains a single, rigid conformation is antithetic to a biologically active protein dependent on conformational plasticity to interact with other molecules and/or for catalytic activity. Unsurprisingly, proteins exist as an ensemble of conformations that constitute the native state. Although this permits proteins to be biologically active, it renders a protein sensitive to environmental perturbations, such as heat stress, that favour non-native aggregation-prone conformers. Prolonged exposure to mild elevated temperatures and short exposure to extreme temperatures cause cell toxicity. Differential scanning calorimetry of whole cells suggests that denaturation of approximately 7-20% of the proteome correlates with the onset of cell death, suggesting that the cause of toxicity correlates with the loss of protein function due to denaturation and aggregation (Lepock 2005). These intracellular aggregates can be observed as amorphous electron-dense anomalies in transmission electron micrographs of yeast cells exposed to severe heat shock (Parsell et al. 1994). Although these thermolabile targets are currently unknown, it is likely that they are crucial cell survival factors.

The protein aggregates formed within cells contain not only denatured protein but also a large quantity of native protein (Borrelli et al. 1996). For example, relatively stable reporter proteins become insoluble due to aggregation when introduced into mammalian cells and heated at temperatures producing thermal damage (Nguyen et al. 1989). This can be explained by the denaturation of endogenous thermolabile proteins in cells during heat shock which then act as nucleation sites for the aggregation of other, non-denatured proteins. The amount of native protein in heat-induced aggregates appears to be greater than the amount of denatured protein (Borrelli et al. 1996). Thus,
denaturation and subsequent aggregation in cells is a complex event involving more than just the thermolabile proteins denaturing during heat shock.

1.3.1 The heat shock response

Cells respond to thermal stress by two basic mechanisms: inactivation of protein function and activation of signaling pathways. The former is caused by protein denaturation and aggregation while the later induces the upregulation of a number of genes to promote survival under stress conditions to combat the lethal effect of protein aggregation. The protein products of these genes are termed heat shock proteins. Molecular chaperones constitute a large proportion of these gene products and help prevent protein aggregation using the same mechanism that is used to assist in protein folding under non-stressed conditions (Hsp70/40, sHsps). Finally, proteins required for the synthesis of the disaccharide trehalose, which promotes general protein stability, are expressed in high quantities.

1.3.2 Trehalose, a chemical chaperone

Trehalose, a carbohydrate composed of two glucose molecules attached via their anomeric carbons, is found in a great variety of species, most notably in anhydrobionts such as brine shrimp, nematodes, and baker’s yeast (Singer and Lindquist 1998). These unusual organisms are also called ‘resurrection plants’ because of their ability to withstand almost complete desiccation. They endure long periods of seemingly suspended animation and return from dormancy to full activity once water becomes available again. Although barely detectable in log-phase yeast, trehalose accumulates to remarkably high levels – up to 20% of the dry weight of the cell – in stationary phase cells and spores, as well as in log-phase cells that have been exposed to high
temperatures, suggesting that trehalose is a potent stress tolerance factor. Under these conditions, trehalose expression is restricted to nuclear and cytoplasmic regions of the cell, accumulating to approximately 0.5 M. Such high levels of sugar, it was suggested, would most certainly alter the structural properties of the constituents of the nucleus and the cytoplasm. Indeed, it was later shown in-vitro that high concentrations of trehalose extend the range of temperatures over which proteins retain their native state and that trehalose levels correlate with cell survival under adverse conditions in-vivo (Singer and Lindquist 1998).

Trehalose is synthesized constitutively at extremely low levels by an enzymatic complex consisting of Tps1p, Tps2p, Tps3p, and Tsl1p. Tps1 and tps2 mutants exhibit decreased survival after extreme heat treatment (reviewed in (Singer and Lindquist 1998)). The activity of this complex increases with heat, but it is unclear whether this is influenced by phosphorylation of the complex. Each component of the complex is expressed constitutively, and the expression of TPS1 and TPS2 is further induced by heat, further increasing the activity of the enzyme complex.

Trehalose protects cells from thermal damage in two ways: stabilization of membrane integrity and protection of native proteins from heat damage (reviewed in (Crowe 2007). To maintain membrane structure, it was hypothesized that under stress conditions trehalose delays the breakdown of phase-separated membrane microdomains (lipid rafts) due to increased mobility in lipid bilayers (Crowe 2007). Lipid rafts contain localized membrane-bound proteins involved in a variety of crucial cellular processes including signaling and endocytosis, and preservation of these functions after thermal insult is critical to cell survival.
Trehalose also protects cells from heat by stabilizing proteins at high temperatures. Experiments using cells expressing temperature-sensitive reporter proteins indicated that enzymes are better able to retain activity after heat shock in cells producing trehalose (Singer and Lindquist 1998). Trehalose is able to prevent native proteins from denaturing and failing this, can also suppress the aggregation of thermolabile proteins in vitro, a property thought to reside exclusively with Hsps (Singer and Lindquist 1998). Proteins protected from aggregation by trehalose were found to be in an inactive and partially unfolded state (Singer and Lindquist 1998). However, trehalose stabilization of unfolded proteins competitively inhibits the molecular chaperone machinery from reactivating these substrates, suggesting that a mechanism for substrate release from trehalose is required prior to chaperone action (Singer and Lindquist 1998). Indeed, trehalose is degraded soon after a stress has passed, far more rapidly than are Hsps (Hottiger et al. 1987; Hottiger et al. 1987). Furthermore, mutants lacking an ability to degrade trehalose during recovery after heat shock are thermosensitive (Nwaka et al. 1995; Nwaka et al. 1996). These observations have led to a combinatorial model for thermotolerance that describes an intimate interplay between trehalose and the molecular chaperone machinery of the cell (Singer and Lindquist 1998).

During conditions of extreme or prolonged stress, the anti-aggregation capacity of the cell contributed by both trehalose and Hsps can be overwhelmed, leading to protein aggregation and cell death. For cells to survive, a mechanism to resolve these toxic aggregates is therefore required. This role is uniquely filled by a novel molecular chaperone that is the main subject of this thesis.
1.4 An introduction to Hsp104

1.4.1 Biological functions of Hsp104

1.4.1.1 Thermotolerance

Cells can deal with the dispersal of aggregated proteins by degrading them and recovering their amino acid building blocks for the synthesis of new proteins. This approach is not sufficient to ensure cell survival since proteins critical for viability can themselves be aggregated or otherwise inactivated by sequestration into protein aggregates. This is exacerbated by an almost complete halting of protein translation so that these critical targets cannot by replaced by de novo protein synthesis. However, in yeast an alternative pathway of aggregate clearance involves the reversal of protein aggregation by extracting polypeptides from aggregates accompanied by refolding of those polypeptides. The recovery of full-length active proteins in this manner minimizes the energy consumption and time devoted to the synthesis of replacement proteins during recovery from acute stress.

Central to this recovery pathway is the ATPase heat shock protein 104 (Hsp104). Yeast cells lacking Hsp104 exhibit a profound defect in thermotolerance because Hsp104’s contribution to survival following extreme heat shock is unrivalled by any other molecular chaperone in the yeast arsenal (Sanchez and Lindquist 1990). The sequence similarity of Hsp104 to the ATPase components of the newly-described caseinolytic proteases (Clps) in bacteria (Squires and Squires 1992) initially suggested that Hsp104 promotes survival in severely heat-shocked cells by promoting the degradation of misfolded proteins. Shortly thereafter, it was observed that the electron-dense material formed by severe heat shock in yeast was dispersed during recovery in
wild type cells but persisted in cells lacking Hsp104 (Parsell et al. 1994). Furthermore, a heterologously-expressed bacterial luciferase-fusion protein that lost activity and solubility rapidly during heat shock was not protected from aggregation nor was degraded in cells expressing Hsp104, but was resolubilized and reactivated, suggesting a novel pathway for aggregate clearance existed that had yet to be described.

1.4.1.2 Prion propagation

Yeast prions represent a class of aggregation-prone proteins that spontaneously form highly ordered polymers under non-stress conditions (Wickner et al. 2000). Prion aggregates are physically distinct from the amorphous aggregates that form during heat stress. Yeast prion proteins serve as the heritable unit for yeast prion transmission from mother to daughter cells during mitosis and meiosis and are analogous to self-propagating aggregates of the mammalian prion protein that spread transmissible spongiform encephalopathies (TSEs) from diseased to healthy animals (Wickner et al. 2004; Wickner et al. 2007). Yeast prion proteins have in common an amyloid-forming domain enriched in asparagine and glutamine residues and are necessary and sufficient for fibre formation and the faithful transmission of the prion state. There are at least three well-characterized S. cerevisiae prions: [PSI+], [URE3] and [RNQ+] (also known as [PIN+]). Each of them can form self-propagating aggregates composed of the proteins Sup35, Ure2, and Rnq1 respectively.

Prion proteins spontaneously form fibrillar aggregates in vitro (Glover et al. 1997; Taylor et al. 1999; Patel and Liebman 2007). Pre-existing fibres of a prion protein act as seeds that extend indefinitely by the recruitment of additional soluble protein onto the fibre ends (Scheibel et al. 2001). Since the partitioning of seed particles between mother
and daughter cells is required for stable inheritance, a mechanism must exist for
generating new seeds from existing fibres. For [\(PSI^+\)], [\(URE3\)] and [\(RNQ^+\)], elimination
of Hsp104 by the deletion of its gene results in the loss of the prion (Moriyama et al.
2000; Wegrzyn et al. 2001). It was originally postulated that, given Hsp104’s role in
remodeling and refolding amorphous aggregates when it is highly expressed in heat
shocked cells, the relatively low level of Hsp104 expressed in unstressed cells is
sufficient to fragment long prion fibres to produce smaller amyloid particles that can be
readily transmitted through the transfer of cytoplasm to daughter cells and spores, and
that overexpression of Hsp104 destabilizes prions by complete dispersal of the aggregates
(Kushnirov and Ter-Avanesyan 1998). The [\(PSI^+\)] prion is indeed eliminated by the
overexpression of Hsp104 (Chernoff et al. 1995). However, the ability of Hsp104 to cure
prions cannot be generalized; Hsp104 overexpression does not cure cells of [\(URE3\)]
(Moriyama et al. 2000) or [\(RNQ^+\)] (Derkatch et al. 1997).

1.4.1.3 Asymmetric distribution of oxidatively damaged proteins

In addition to dispersing heat-induced aggregates and propagating prions, Hsp104
also plays a role in protecting proteins from oxidative damage. Several lines of evidence
suggest this. First, it was observed early on that Hsp104 contributes enormously to
thermotolerance in aerobic culture but has a minimal contribution to thermotolerance
under anaerobic conditions (Davidson et al. 1996). Secondly, Hsp104 confers enhanced
survival in cells exposed to oxidative stress induced artificially by treatment with
hydrogen peroxide and menadione (Ueom et al. 2003). Finally, Hsp104 may also play a
role in diminishing the effects of cumulative protein damage associated with aging in
yeast. During cytokinesis, mother cells sequester aggregates of carbonylated proteins in a
process that is dependent on the SIR2 gene (Aguilaniu et al. 2003) and cells lacking Sir2 show decreased life span. Deletion of HSP104 resulted in a breakdown of damage asymmetry while overproduction of Hsp104 suppressed the accelerated aging of cells lacking Sir2. Hsp104 was also observed to colocalize with carbonylated proteins generated by treatment with hydrogen peroxide (Erjavec et al. 2007). More recently, Hsp104-GFP fusion proteins were observed to comigrate with protein aggregates out of daughter cells by an as yet uncharacterized retrograde transport mechanism (Liu et al. 2010). It is not yet clear how the action of Hsp104 in resolubilizing aggregated proteins can diminish the toxic effects of protein oxidation and facilitate the asymmetric distribution of oxidatively damaged proteins.

1.4.2 Regulation of Hsp104 expression

Unlike the other molecular chaperones that are abundant even in unstressed cells and that perform essential functions, Hsp104 is present at relatively low levels in cells and ΔHSP104 cells have no observable phenotype when grown under optimal conditions. A global analysis of protein expression in S. cerevisiae (Ghaemmaghami et al. 2003) indicates that Hsp104 is expressed at 3.24 X 10⁴ molecules/cell while Hsp90 (Hsp82 and Hsc82 combined) is at 5.77 X 10⁵ and Hsp70 (Ssa1 and Ssa2 combined) is at 6.33 X 10⁵ molecules/cell. However, the expression of Hsp104 is upregulated by a number of stresses, and in respiring and stationary phase cells (Sanchez and Lindquist 1990; Sanchez et al. 1992).

Induction of HSP104 is governed by the transcription factors Hsf1, Msn2, and Msn4 (Grably et al. 2002). Hsf1, a protein that is evolutionarily conserved from yeast to humans, is required for cell viability at physiological temperatures (reviewed in (Pirkkala
et al. 2001). Hsf1 binds to heat shock elements (HSEs) within the promoter of target genes, including HSP genes. The activity of Hsf1 is induced by a variety of stresses including heat, ethanol treatment, oxidative stress, and glucose starvation. Under physiological conditions, Hsf1 binds to promoter elements with low affinity and with high affinity under stressed conditions. It has been suggested that this switch to an activated form is controlled by hyperphosphorylation of Hsf1 upon initiation of the stress (Hietakangas et al. 2003; Guettouche et al. 2005). Alternatively, Hsp90 binding to Hsf1 under physiological conditions might repress its translocation to the nucleus thereby preventing activation of transcription of HSE containing genes (Zou et al. 1998). Upon induction of stress, Hsp90 may competitively bind to misfolded proteins releasing Hsf1 for activation of transcription.

Msn2 and Msn4 (Msn2/4) are functionally redundant zinc-finger containing transcription factors that regulate the general stress response and, unlike Hsf1 and Hsf2 which are activated by few stresses, are induced by a much broader range of stresses that include heat shock, oxidative stress, osmotic shock, and glucose starvation (Martinez-Pastor et al. 1996). Msn2/4 bind to stress response elements (STREs) in promoter regions of target genes (Schmitt and McEntee 1996) and hyperphosphorylation of Msn2/4 may be required for their activation (Garreau et al. 2000). Many of these genes overlap with Hsf1 targets and include most Hsps (Treger et al. 1998; Hahn et al. 2004). The STRE system can therefore be thought of as a general non-specific stress response system.

The HSP104 gene contains two HSEs and three STREs and all of them are cooperatively required for maximal inducible expression (Grably et al. 2002). Each
system of transcriptional regulation can induce Hsp104 expression alone, backing up the other. A mutant carrying a deletion of the HSEs and STREs in the HSP104 gene is incapable of inducing the expression of Hsp104 and phenocopies the thermotolerance defect of an Hsp104 deletion mutant (Treger et al. 1998; Amoros and Estruch 2001).

Hsp104 induction after heat shock exhibits a unique pattern of delayed upregulation (DUR) (Seppa et al. 2004). When yeast cells grown at a physiological temperature of 24°C, preconditioned at 37°C, and treated briefly at 50°C were shifted back to 24°C, Hsp104 expression was negligible for 1 h and was maximally induced after 3 h of recovery, returning to basal levels after 5 h (Seppa et al. 2004). HSEs were necessary and sufficient for DUR while STREs were dispensable (Seppa et al. 2004). However, deletion of Msn2/4 transcription factors delayed and reduced DUR of Hsp104 indicating that there is at least some functional overlap between the two transcriptional regulation systems (Seppa et al. 2004).

DUR is slow and is a characteristic of at least several other molecular chaperones (Bip/Kar2, LHS1, Hsp78), but the recovery of major cell functions after thermal insult is even slower (Seppa and Makarow 2005). Major cellular functions are abolished by severe heat stress at 48 to 50°C. After a shift of the cells to a physiological temperature, these functions do resume, slowly but efficiently. ATP production resumes in about 4 h. Protein synthesis is normal after 4 to 6 h, and cell division is normal after 20 h (Jamsa et al. 1995; Hanninen et al. 1999; Simola et al. 2000; Seppa et al. 2004). The inhibition of these functions is likely due to protein denaturation, followed by reactivation due to conformational repair performed by chaperones. DUR of chaperones is likely part of the molecular mechanism ensuring the survival of cells after severe heat stress by producing
excessive levels of chaperones during recovery from thermal insult to mediate repair of heat-denatured proteins. Because unicellular organisms cannot rely on apoptosis to remove damaged cells, survival is dependent on the conformational repair of denatured proteins. However, it is unclear whether all molecular chaperones exhibit this brief temporal delay in upregulation and how DUR contributes to cell survival.

1.4.3 Proposed Hsp104 structure

1.4.3.1 Domains of Hsp104

Hsp104 is a member of the Hsp100/Clp subfamily of AAA+ ATPases (Neuwald et al. 1999). Hsp100/Clp proteins were originally identified as the ATP-dependent regulators of proteolysis in bacteria (Squires and Squires 1992; Schirmer et al. 1996). AAA+ superfamily members consist of a diverse group of proteins that form ring-shaped hexamers. Each Hsp100/Clp subunit is composed of at least one AAA+ module consisting of a nucleotide binding domain (NBD) and an α-helical small domain (SD) (Erzberger and Berger 2006). Hsp104 contains two AAA+ modules (denoted AAA+1 and AAA+2), and both are required in order for Hsp104 to function in thermotolerance (Fig 1.2) (Parsell et al. 1991; Hattendorf and Lindquist 2002).

Hsp104 and ClpB, its bacterial ortholog, are unique among the Hsp100/Clp family because the SD of AAA+1 contains an insertion that is predicted to form a coiled-coil domain (CCD). No function has yet to be assigned to CCD (also known as the “spacer” or “middle” region). However, screens for dominant negative alleles of Hsp104 (Schirmer et al. 2004) recovered mutants containing amino acid substitutions in the coiled-coil region demonstrating that this domain does indeed have a significant role in Hsp104 function.
Figure 1.2 Domain structure of Hsp104. Hsp104 consists of five parts: two AAA⁺ modules (AAA⁺1 and AAA⁺2) with each module consisting of a nucleotide binding domain (NBD) and an α-helical small domain (SD), an N-terminal domain (NTD), a coiled-coil domain (CCD) inserted into AAA⁺1 and a C-terminal extension (CTE). Amino acid substitutions in the Walker A motifs (K218 and K620) diminish ATP binding while substitutions in the Walker B motifs (E285 and E687) abolish ATP hydrolysis. Non-conservative substitutions in pore loops or diaphragms of AAA⁺1 (KYKG) or AAA⁺2 (GYVG) do not affect ATP binding or hydrolysis but abolish thermotolerance.

Hsp104 also has an N-terminal domain (NTD) consisting of 152 amino acid residues. In other Hsp100s, the NTD plays a role in substrate binding and selection by functioning as a binding site either for substrates or for adaptor proteins that themselves bind to substrates and deliver them to Hsp100s. The NTDs of ClpX and ClpA are required for the efficient delivery of proteins bearing an SsrA tag - an 11-amino acid degradation signal that is co-translationally added by the tmRNA system in bacteria to nascent polypeptides on stalled ribosomes - by the adaptor proteins SspB and ClpS, respectively (Guo et al. 2002; Zeth et al. 2002; Dougan et al. 2003; Wah et al. 2003). The N-terminal domain of ClpB appears to influence the interaction of ClpB with protein aggregates (Liu et al. 2002; Tanaka et al. 2004; Barnett et al. 2005) and may enhance chaperone function (Chow et al. 2005; Nagy et al. 2009). The N-terminal domain of Hsp104, however, is not essential for thermotolerance or for prion propagation but is
required for curing of $[\text{PSI}^+]$ by overexpression of Hsp104 (Hung and Masison 2006) indicating that the NTD indeed contributes to Hsp104’s protein remodeling function.

Finally, Hsp104 has a C-terminal extension (CTE) for which several roles have been assigned. Deletion of the last 38 amino acids of Hsp104 diminishes its solubility at high temperature in vivo and its assembly in vitro suggesting that the extension has an important structural role in stabilizing the protomer and the hexamer (Mackay and Glover, Biochem, 2008). The CTE is also required for poly-L-lysine to interact with and stimulate the ATPase activity of Hsp104, suggesting that substrate binding to the CTE may allosterically influence nucleotide hydrolysis in the AAA$^+$ modules (Cashikar et al. 2002). Fungal Hsp104s and plant Hsp101s have CTEs that contain short acidic extensions that are not present in bacterial orthologs. In S. cerevisiae Hsp104, this extension consists of residues 870-908. The extreme acidic tetrapeptide (DDLD, residues 905-908) resembles the EEVD motif found in cytosolic members of the Hsp70 and Hsp90 chaperone families and is known to serve as a docking site for tetratricopeptide-repeat (TPR)-containing cochaperones (such as the Hsp-organizing protein, HOP) (Chen and Smith 1998). Indeed, Hsp104 interacts with the Hsp90 cochaperones Sti1, Cpr7, and Cns1 in vitro and in vivo in respiring cells and deletion of the last 8 amino acids of Hsp104 (including the DDLD motif) abolishes the interaction with Sti1 (Abbas-Terki et al. 2001).

1.4.3.2 Proposed three-dimensional structure of Hsp104

To date, no crystal structures of Hsp104 have emerged, perhaps due to the dynamic nature of the protein. However, structures of native hexamers of HslU, an Hsp100 member with a single AAA$^+$ module, have been solved by several groups
(Bochtler et al. 2000; Sousa et al. 2000; Wang et al. 2001). Monomeric structures of
other Hsp100s including ClpX (Kim and Kim 2003), ClpA (Guo et al. 2002), and of the
bacterial Hsp104 ortholog ClpB (Lee et al. 2003), have also been obtained and have been
used to reconstruct models of their respective hexamers. The reconstruction of
hexameric ClpB gives insight into the quaternary structure of Hsp104. This model shows
that ClpB forms a two-tiered hexameric ring in which the two AAA\(^+\) modules in each
protomer stack on top of one another (Fig 1.3). The coiled-coil linker domain consists of
a long and mobile coiled coil that is located on the outside of the hexamer (Lee et al.
2003). At the centre of the hexamer is a narrow axial channel that is analogous to what
has been observed in EM images of ClpX (Ortega et al. 2000) and ClpA (Ishikawa et al.
2001). These features have been confirmed by cryo-EM images of ClpB (Lee et al.
2003; Lee et al. 2007). However, no cryo-EM density corresponding to the tips of the
ClpB linker region was observed suggesting that they are flexible. Likewise, the NTDs
of ClpB are also poorly resolved suggesting that these too are flexible.

To gain insight into the three-dimensional structure of Hsp104, we used structural
data from ClpB to generate a homology model of the Hsp104 hexamer (Fig 1.4A). This
reconstruction of Hsp104 consists of a model that is two-tiered in which the two AAA+
modules in each protomer are stacked on top of one another. These general features are
consistent with cryo-EM images of ClpB (Lee et al. 2003; Lee et al. 2007) and recent
cryo-EM reconstructions of the Hsp104 hexamer fitted with the crystal structure of the
ClpB monomer (Lee et al. 2010). Crucially, a narrow axial channel penetrates the centre
of the hexamer and conserved loops that are crucial for the binding and unfolding of
substrates (see Chapters 3 and 4) line this channel (Lee et al. 2010). The coiled-coil
Figure 1.3 Reconstruction of the ClpB hexamer. (A) Cut-away side view of the three-dimensional cryo-EM reconstruction with the structure of the ClpB hexamer docked in. The N-terminal domains and part of the ClpB/Hsp104-linker are not visible in the 3D reconstruction because they are mobile. AMPPNP molecules are shown as space-filling CPK models and are colored in red (AAA+1) and blue (AAA+2), respectively. (B) Cut-away top view of AAA+1. (C) Cut-away top view of AAA+2. (D) Surface representation of the ClpB-AMPPNP hexamer. Each ClpB subunit is colored differently. The secondary structure elements of one ClpB monomer are shown underneath the semitransparent surface. Adapted from (Lee et al. 2004).
domains emerge from the small domain of the first AAA⁺ module and are displayed on the exterior of the hexamer (Lee et al. 2003). Intriguingly, no additional masses on the outside of the Hsp104 hexamer could be attributed to coiled-coil domains in these cryo-EM images, suggesting that this domain could indeed be mobile (Lee et al. 2010). To address this, an Hsp104 chimera was constructed in which T4 lysozyme, a small stably folded protein of known structure, was inserted in the middle of the coiled-coil domain. The structure of this chimera was examined by cryo-EM and showed additional mass densities on the outside of the hexamer surrounding AAA⁺1. These additional mass densities were not observed in the wild type Hsp104 hexamer and, therefore, can be attributed to the folded lysozyme moiety. In this study, the domain arrangement of Hsp104 is consistent with ClpB as well as with other AAA⁺ machines.

Recently, a cryo-EM study of Hsp104 bound by ATPγS (Wendler et al. 2007) revealed a dramatically different picture of Hsp104 structure compared to the aforementioned models (Fig 1.4B). First, in contrast to the narrow axial channel suggested by ClpB-based model, Hsp104 in the most recent model forms a large central cavity up to 78 Å in diameter with the N-domains forming a well resolved cap over the central cavity. Second, fitting of the coiled-coil domains to electron density remaining after fitting of the AAA⁺ modules indicated that these arms intercalate between adjacent subunits where they form part the walls of the central cavity and disrupt the domain interactions that are typical of all other AAA⁺ proteins (see 1.4.4.3). Third, rather than stacking directly on top of one another, the AAA⁺1 module of one subunit is situated almost directly on top of the AAA⁺2 module of the adjacent subunit.
**Figure 1.4 Structural models of Hsp104.** (A) Model of Hsp104 structure based on ClpB. A homology model (Guex and Peitsch 1997; Schwede et al. 2003) was created using the deposited data for *T. thermophilus* ClpB (1QVR) (Lee et al. 2003) and a hexameric model (Diemand and Lupas 2006) was created using the NSF hexamer (1NSF). (B) Model of Hsp104 based on cryo-EM. Coordinates for the structure of Hsp104 fitted to cryo-EM density maps were kindly provided by P. Wendler. The N-terminal domains were omitted for comparison with the ClpB-based model. Images were rendered with POV-Ray (http://www.povray.org).
To date, it is still unclear as to which of the two structural models accurately represents the Hsp104 hexamer. Additional structural and biochemical data is required to distinguish between these two radically different models.

1.4.3.3 ATPase activity of Hsp104

As in many AAA+ members that hydrolyze ATP to perform some mechanical function, Hsp104 converts energy from ATP hydrolysis into work in the form of protein remodeling. Initial studies in which nucleotide binding to each NBD was inhibited by substitution of conserved Lys residues in Walker A motifs of each AAA module (Lys 218 and Lys 620) (Fig 1.2) with Thr indicated that both NBDs are required for Hsp104 to provide thermotolerance (Parsell et al. 1991). The conserved residues of the Walker A motif are proposed to be important in binding the β- and γ-phosphates of the bound ATP and Mg$^{2+}$ ion, respectively (Snider and Houry 2008). Interestingly, Hsp104’s basal ATPase activity was predominantly a reflection of the activity of NBD1 (Schirmer et al. 1998) while NBD2 has a predominant role in stabilizing nucleotide-dependent assembly of Hsp104 (Parsell et al. 1994). Mutants of Hsp104 that fail to properly assemble also exhibit impaired ATP hydrolysis activity (Schirmer et al. 2001; Tkach and Glover 2004). In a hexameric model of Hsp104 based on ClpB, the predominant effect of oligomerization on Hsp104s ATPase activity is explained in part by conserved structural elements in the ATP binding site of AAA+ proteins. A conserved arginine residue, known as an “arginine-finger,” is oriented such that it projects into the neighbouring subunit and interacts with the γ-phosphate of the ATP molecule bound to the adjacent AAA+ module (Karata et al. 1999). However, in the cryoEM reconstruction of Hsp104 from the Saibil group, these “arginine finger” motifs are displaced by the intervening
coiled-coil. Furthermore, substitution of conserved arginines in the coiled-coil reduce Hsp104’s ATPase activity suggesting a possible catalytic role for this domain in ATP hydrolysis (Wendler et al. 2007). Nevertheless, correct assembly of Hsp104 will clearly have an impact on this important catalytic interaction. Additionally, mutations in the hydrophobic core of SD2 in AAA¹² (Tkach and Glover 2004) and deletion of the C-terminal extension (Mackay et al. 2008) inhibit assembly and consequently the ATPase activity of Hsp104 supporting the role of AAA¹² in oligomerization.

The Walker B motifs of AAA⁺ NBDs contain an acidic residue (Glu 285 and Glu 687 in NBD1 and NBD2, respectively) (Fig 1.2) that is predicted to act as a catalytic base, abstracting a proton from a molecule of water and thereby priming it for a nucleophilic attack on the γ-phosphate of a bound ATP molecule (Snider and Houry 2008). Non conservative substitutions in these residues result in a mutant that is locked into an ATP bound state without hydrolysis. This ‘trap’ mutant is capable of binding unfolded protein (reduced carboxymethylated α–lactalbumin (RCMLα), an unfolded soluble protein). Such an interaction can only be observed in the wild type protein with addition of the slow-hydrolyzing ATP analogue ATPγS (Bosl et al. 2005). Equilibrium binding of an unfolded protein to Hsp104 requires only NBD1 to be locked in an ATP bound state (Schaupp et al. 2007). Additionally, elimination of hydrolysis at NBD1, which dominates the ATPase activity of the wild type protein, revealed that ATP hydrolysis at NBD2 is strongly accelerated in the presence of unfolded protein (Schaupp et al. 2007).
Another conserved interaction between an amino acid side chain and the bound nucleotide is observed with the sensor-1 motif. This is a polar residue that interacts with the $\gamma$-phosphate of ATP and substitution at this position in NBD2 of Hsp104 (N728A) locks this domain in an ATP bound state (Hattendorf and Lindquist 2002) and diminishes the $k_{cat}$ of hydrolysis at NBD1 by four-fold. This suggests ADP binding at NBD2 and therefore hydrolysis likely accelerates ATP turnover at NBD1. Thus, NBD2 exerts a strong allosteric influence on the steady-state kinetic behaviour of NBD1. Taken together, these observations imply that stable substrate binding at AAA$^+$1 stimulates hydrolysis at NBD2 and that increased occupancy of ADP in NBD2 could, in turn, enhance ATP turnover at NBD1.

1.4.4 Proposed Hsp104 reaction cycle

1.4.4.1 Chaperone networks and Hsp104

A trademark characteristic of molecular chaperones is their ability to interact with unfolded proteins and thereby prevent them from aggregating. Surprisingly, in in vitro aggregation assays, Hsp104 displayed no such capability, thereby distinguishing its action from that of other molecular chaperones (Glover and Lindquist 1998). Instead, Hsp104 forms a unique chaperone system that requires Hsp70 together with an Hsp40 partner to resolve previously aggregated proteins in a two-step process. First, aggregates are disaggregated in an Hsp104-dependent step by continuous extraction of unfolded polypeptide molecules (Schlieker et al. 2004). Second, unfolded polypeptides refold to the native state in an Hsp70/40-dependent refolding phase (Goloubinoff et al. 1999). The synergy of these two distinct molecular chaperone machineries led to the coining of the term “bichaperone network” to describe this functional interaction (Goloubinoff et al. 1999).
A functional interaction between Hsp104 and small heat shock proteins has also been shown. *In vitro*, Hsp104, Ssa1, and Ydj1 reactivate luciferase-Hsp26 co-aggregates 20-fold more efficiently than luciferase aggregates alone suggesting that small heat shock proteins may render aggregates more accessible to Hsp104, Hsp70, and Hsp40 (Cashikar *et al.* 2005; Haslbeck *et al.* 2005).

### 1.4.4.2 Substrate remodeling

The exact mechanism by which protein aggregates are resolved by Hsp104 has yet to be determined. Based on biochemical and structural data, several models have been proposed to explain the features of Hsp104-mediated protein remodeling.

#### 1.4.4.2.1 The crowbar model

In the “molecular crowbar” model, Hsp104 makes multivalent contacts in the target aggregate. Upon ATP hydrolysis the Hsp100 oligomer undergoes a conformational change that disrupts contacts between aggregated proteins (Fig 1.5 A). Hydrophobic surfaces exposed in this manner can be accessed by other chaperones that can assist in the refolding of the polypeptide.

Hsp104 and its bacterial ortholog ClpB both contain coiled-coil or linker domains that are unique to this subfamily of Hsp100s. Structural models of ClpB reveal that this domain forms a two-bladed propeller that rings the exterior of the hexamer (Lee *et al.* 2003). Restriction of the movement of the linker domain by introduction of disulphide bridges abolishes ClpB’s disaggregation activity without compromising ATPase activity or the ability to form hexamers. This suggests that movement of the linker domains of Hsp104 and ClpB may be the conformational change required to remodel aggregated...
protein structures (Glover and Lindquist 1998; Glover and Tkach 2001; Lee et al. 2003). It is therefore attractive to speculate that the coiled-coils function as substrate binding sites and upon binding of a protein aggregate, ATP hydrolysis-dependent movement of the coiled-coils may provide the necessary torque to pry apart protein aggregates revealing hydrophobic sites to which Hsp70 can bind and prevent reaggregation. However, no direct interaction between the coiled-coil and substrate has been detected. Furthermore, a mutant of Hsp104 containing a substitution of the coiled-coil with T4 lysozyme retains disaggregase function indicating that the coiled-coil is not required for substrate binding (Lee et al. 2010). These observations suggest that Hsp104 likely utilizes an alternative method for aggregate resolubilization.

1.4.4.2.2 An unfolding – threading model

In the case of peptidase-associated AAA+ proteins, a mechanism that mediates the unfolding and translocation of bound substrates through the central pore of the protease complex has been proposed (Wang et al. 2001). Playing a key role in this mechanism are conserved aromatic residues that are located at the central pore of AAA+ proteins. These residues may serve as molecular clamps by binding and releasing substrates, thereby preventing the backsliding of polypeptides during the translocation process. It has been further suggested that nucleotide-dependent conformational changes of these residues directly couple substrate translocation to substrate unfolding (Wang et al. 2001). Consistent with such an activity, the corresponding aromatic residues of another Hsp100 member FtsH has been shown to be crucial for substrate degradation (Yamada-Inagawa et al. 2003). Interestingly, the aromatic residues and their relative positioning are
Figure 1.5 Possible mechanisms for aggregate disassembly by Hsp100 chaperones. In the “molecular crowbar” model (A) the Hsp100 hexamer makes multivalent contacts in the target aggregate. Upon ATP hydrolysis the Hsp100 oligomer undergoes a conformational change that disrupts contacts between aggregated proteins. Hydrophobic surfaces exposed in this manner can be accessed by other chaperones (symbolized by the yellow orbs). Repetitions of the cycle will release chaperone-bound monomers that can refold upon release. In the “molecular ratchet” model (B) the Hsp100 may latch on to exposed segments of polypeptide (like an exposed, hydrophobic SsrA tag). Cycles of ATP hydrolysis unfold the protein and permit threading of the polypeptide through a central channel. Clamping and release of flexible loop residues surrounding the axial pore ensure net translocation of the polypeptide. As the polypeptide emerges in an extended conformation (reminiscent of the nascent polypeptide emerging from the ribosome) it will acquire a protective shield of molecular chaperones.
conserved in both AAA domains of Hsp104 and ClpB (Glover and Tkach 2001). Furthermore, these residues appear to be located on mobile loops, as the corresponding regions could not be resolved in the ClpB crystal structure (Lee et al. 2003). Cryo-EM reconstructions of ClpB in the ATP, AMP-PNP, and ADP bound states show that these loops are stabilized at the centre of the channel only in the ATP-activated state providing a structural basis for high-affinity protein binding in the ATP-bound state (Lee et al. 2007). These observations suggest that Hsp104 and ClpB may, like peptidase-associated AAA⁺ proteins, exhibit a translocation activity that could mediate the extraction of an unfolded polypeptide from the aggregate (Fig 1.5 B). Several studies suggest that translocation of single polypeptides through the central pore may be a common feature among AAA⁺ chaperones (Hoskins et al. 2000; Kim et al. 2000; Ortega et al. 2000; Singh et al. 2000; Reid et al. 2001).

1.4.4.3 Substrate recognition

For many members of the Hsp100 family, recognition of specific peptide sequences initiates the protein remodeling process. Protein substrates of ClpXP and ClpAP generally contain recognition signals of roughly 10–15 residues that can be located either at the N- or C- termini (Flynn et al. 2003). These peptide tags physically interact with the Hsp100, targeting the polypeptides for degradation by ClpAP or ClpXP (Haebel et al. 2004). When appended to the N- or C- termini of heterologous proteins, these tags are sufficient to target the fusion protein for recognition and degradation by ClpXP or ClpAP.

Like other Hsp100s, Hsp104 may initially engage substrates by binding to peptides displayed on the surface of aggregated proteins. However, Hsp104 must also
use some mechanism to distinguish between misfolded proteins and their correctly folded native conformers. It is attractive to speculate that Hsp104 may bind preferentially to segments of polypeptides that are normally buried in the native structure and only become solvent-exposed upon misfolding or denaturation. These peptide segments are most likely enriched in hydrophobic amino acids.

1.5 Thesis Rationale and Objective

Hsp104 has a unique ability to reverse protein aggregation and so clearly has a potential usage in therapeutic strategies to combat diseases associated with protein aggregation. To even begin to contemplate this, a thorough understanding of how Hsp104 recognizes and unfolds its substrates is required.

The objective of this thesis is separated into three distinct bodies of work. In the second chapter of this thesis, a translocation activity of Hsp104, as part of the protein disaggregation reaction, and a role for the aromatic residues that line the central channel of the Hsp104 hexamer is investigated. In the third chapter of this thesis, a high affinity peptide that can act as a substrate mimetic is used to explore substrate processing by Hsp104. An allosteric model for protein unfolding is presented. Finally, in the fourth chapter, I analyze elements in both a model peptide and Hsp104 that are required for rapid formation of a substrate-Hsp104 complex.
2 Evidence for an unfolding/threading mechanism for protein disaggregation by *Saccharomyces cerevisiae* Hsp104

Ronnie Lum, Johnny M. Tkach, Elizabeth Vierling, and John R. Glover

Johnny Tkach constructed the bacterial expression plasmids used to recombinantly express Hsp104 mutants that contain substitutions at codon 662. Elizabeth Vierling’s laboratory identified the *Arabidopsis thaliana* mutation encoded by the *hot1-1* allele. I performed all of the other experiments.

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2.1 Abstract

_Saccharomyces cerevisiae_ Hsp104, a hexameric member of the Hsp100/Clp subfamily of AAA$^+$ ATPases with two nucleotide binding domains (NBD1 & 2), refolds aggregated proteins in conjunction with Hsp70 molecular chaperones. Hsp104 may act as a “molecular crowbar” to pry aggregates apart, and/or may extract proteins from aggregates by unfolding and threading them through the axial channel of the Hsp104 hexamer. Targeting Tyr 662, located in a Gly-Tyr-Val-Gly motif that forms part of the axial channel loop in NBD2, we created conservative (Phe and Trp) and non-conservative (Ala and Lys) amino acid substitutions. Each of these Hsp104 derivatives was comparable to the wild type protein in ability to hydrolyze ATP, assemble into hexamers, and associate with heat-shock induced aggregates in living cells. However, only those with conservative substitutions complemented the thermotolerance defect of a Δhsp104 yeast strain and promoted refolding of aggregated protein _in vitro_. Monitoring fluorescence from Trp 662 showed that titration of fully assembled molecules with either ATP or ADP progressively quenches fluorescence, suggesting that nucleotide binding determines the position of the loop within the axial channel. A Glu to Lys substitution at residue 645 in the NBD2 axial channel strongly alters the nucleotide-induced change in fluorescence of Trp 662 and also specifically impairs protein refolding. These data establish that the structural integrity of the axial channel through NBD2 is required for Hsp104 function and support the proposal that Hsp104 and ClpB use analogous unfolding/threading mechanisms to promote disaggregation and refolding that other Hsp100s use to promote protein degradation.
2.2 Introduction

The *Saccharomyces cerevisiae* chaperone Hsp104 and its bacterial homolog, ClpB, are hexameric members of the Hsp100 subfamily of AAA\(^+\) ATPases, which contain two AAA\(^+\) modules per subunit, each with a nucleotide binding domain (NBD1 & 2). Hsp104/ClpB are necessary for thermotolerance *in vivo* and function together with Hsp70/DnaK chaperone systems to refold non-native proteins trapped in aggregates (for reviews see (Ben-Zvi and Goloubinoff 2001; Glover and Tkach 2001; Weibezahn et al. 2004)). Hsp104 has been proposed to act as an ATP-dependent “molecular crowbar” that can pry aggregated proteins apart, permitting other chaperones to gain access to otherwise inaccessible hydrophobic surfaces sequestered in the aggregates, thereby facilitating protein refolding (Glover and Lindquist 1998). This proposed mechanism of action is different from that of other members of the Hsp100 family, including ClpA (Katayama et al. 1988), ClpX (Gottesman et al. 1993; Wojtkowiak et al. 1993) and HslU (Yoo et al. 1996), which form complexes with oligomeric proteases and function to unfold proteins in an ATP-dependent manner for proteolysis. Biochemical and structural analyses (Hoskins et al. 1998; Hoskins et al. 2000; Ortega et al. 2000; Singh et al. 2000) indicate that in these Hsp100s, ATP hydrolysis is coupled to global unfolding of substrates as they are threaded through an axial channel of the Hsp100 oligomer (Weber-Ban et al. 1999; Hoskins et al. 2000; Reid et al. 2001) and translocated into the chamber of the associated protease.

Recent data are consistent with aspects of the “crowbar” model of Hsp104/ClpB action. Hsp104/ClpB contains a coiled coil, or linker domain that is unique to this subfamily of Hsp100 proteins and may be the active site for substrate binding and
remodeling. Structural analysis of *T. thermophilus* ClpB reveals that this domain forms a two-bladed propeller ringing the exterior of the assembled hexamer (Lee *et al.* 2003). Mutants of *Tt* ClpB in which motion of the coiled coil domain was restricted by the introduction of disulfide bridges could not facilitate protein refolding, although they had normal ATPase activity and assembly properties. Deletion of this domain also compromises *E. coli* ClpB refolding activity *in vivo* and *in vitro* (Mogk *et al.* 2003). However, while potential action of the coiled coil as a crowbar, unique to Hsp104/ClpB, is appealing, direct interaction of this domain with substrate has not been shown, and movement of the coiled-coil domains as part of a threading/unfolding mechanism, analogous to that used by other Hsp100 proteins, has not been ruled out.

Based on the crystal structure of HslU (Sousa *et al.* 2000; Wang *et al.* 2001), the aperture of the Hsp100 protein conducting channel is thought to be modulated by the position of a flexible loop containing a Gly-Tyr-Val-Gly (GYVG) motif. This loop motif is a conserved feature of Hsp100s with a single AAA⁺ module (Class 2), and in the second AAA⁺ module of Hsp104/ClpB and other Hsp100s with two AAA⁺ modules (Class 1) (see Fig 2.1A). The deployment of the GYVG loop within the channel is fine-tuned by the nucleotide-bound status of HslU (Wang *et al.* 2001). A mechanistic model for HslU protein remodeling was proposed whereby the unfolding of a substrate protein by the “I”-domains, a structural element that projects toward the substrate entry side of the HslU hexamer, is synchronized with the constriction and dilation of the axial channel determined primarily by the position of the GYVG loop (Wang *et al.* 2001). During cycles of ATP-binding and hydrolysis an extended polypeptide in the channel would be transiently arrested and released producing a net motion toward the exit side of the axial
Figure 2.1 The role of conserved axial channel Tyr residues in Hsp104-mediated thermotolerance.

(A) The primary sequences of NBD1 and NBD2 of Class1 Hsp100s, S. cerevisiae Hsp104, T. thermophilus ClpB and E. coli ClpA, as well as the NBD of Class 2 Hsp100s H. pylori ClpX and E. coli HslU are illustrated. Secondary structural elements determined by x-ray crystal structure analysis of ClpB (Lee et al. 2003), ClpA (Guo et al. 2002), ClpX (Kim and Kim 2003) and HslU (Sousa et al. 2000) are indicated below the sequences (arrows = beta-strand, rectangles = helix, solid line = turn or coil, dotted line = unresolved in the structure). Walker A and B motifs and proposed Diaphragms 1 and 2 are boxed. Asterisk indicates Glu645 in Hsp104. (B) A yeast strain lacking Hsp104 was transformed with an empty vector (CON) or vectors for the expression of Hsp104WT, Hsp104Y257A, or Hsp104Y662A. Survival was calculated as the ratio of the number of colony formed by cultures after the indicated times at 50°C to the number of colonies formed by the same cultures receiving a 37°C pre-treatment only. (C) Cell free extracts of cells used in thermotolerance experiments in (B) were analyzed by Western blot with anti-Hsp104 antiserum (top panel). Coomassie blue staining of duplicate SDS-PAGE gel verifies equal protein loading in each lane (bottom panel; CB).
channel and into the entry pore of the HslV proteolytic complex. To determine if the axial channel plays a significant role in Hsp104-mediated disaggregation and refolding, we constructed novel Hsp104 mutants with conservative and non-conservative amino acid substitutions in the Tyr residue of the GYVG motif (Tyr 662). The non-conservative substitutions (Y662A, Y662K) abolish the ability of Hsp104 to refold aggregated protein in vitro and to provide thermotolerance in vivo, although assembly of the hexamer and basal ATPase activity were essentially unimpaired. In contrast, when Tyr 662 was replaced with other aromatic amino acids (Y662W, Y662F) Hsp104 was functional, providing levels of thermotolerance close to that of the wild type protein. Trp 662 then served as a unique fluorescent probe for nucleotide-driven conformational changes within the Hsp104 axial channel. Another substitution in the axial channel, Glu 645 to Lys, also specifically impaired Hsp104 function in refolding and thermotolerance, and also affected the fluorescence signal obtained from Trp 662. In total, these results strongly support the idea that the functions of Hsp100s in either disaggregation or protein degradation share a common mechanism involving the axial channel.

2.3 Materials and methods

2.3.1 Hsp104 mutagenesis

To create the E645K mutation, two segments of DNA were amplified from a pBSKII+ cloning vector containing the SalI/SpeI segment of Hsp104b (Schirmer and Lindquist 1998) using a T7 promoter primer together with the primer E645K(A) and a T3 promoter primer together with E645K(B) (Table 2.1). The PCR product from the (A)
primer pair was digested with SalI and HindIII and from (B) with SpeI and HindIII and were ligated together into SalI/SpeI-digested pBSKII+. The assembled DNA segment was excised from the vector with SalI and SpeI. Y662X (where X= A, K, F, and W) mutants were made by amplifying the same plasmid with the indicated primers together with a T7 promoter primer. PCR products were digested with KpnI and SpeI for subcloning. The Y257A mutation was constructed by the Quickchange (Stratagene) method using the pBSKII+ cloning vector containing the EagI/SacI segment of Hsp104 and primers Y257A(A) and Y257A(B). Mutagenized plasmids were screened for the presence of a StyI site. The expected sequences of all HSP104 segments were confirmed by DNA sequencing and were subcloned into pLA28SX104b (pRS313 backbone (Sikorski and Hieter 1989); CEN/ARS, HIS3, Gal1-10 promoter) for expression in yeast.

<table>
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<th>Mutation</th>
<th>Primers</th>
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<td>E645K(B)</td>
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<td>Y257A(A)</td>
<td>5’-GCATTAACCGCAGTGCCAAGGCCAAAAGGTTGATTTGGAAG-3(^c)</td>
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<tr>
<td>Y257A(B)</td>
<td>5’-CTTCGAAATCACTTTGCCCTTGGCCACCTCGGTTAATGC-3(^c)</td>
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underlined = restriction endonuclease sites
shaded = a, codon 662; b, codon 645; c, codon 257
2.3.2 Protein purification

DNA segments encoding full length Hsp104s were subcloned into pPROEX-HT-b (Life Technologies) that adds an N-terminal 6-His tag followed by a Tobacco Etch Virus (TEV) protease cleavage site. Proteins were expressed in BL21Codon Plus cells (Stratagene). Cells were grown to an optical density of 0.6 at 600 nm in Circlegrow (MP Biomedicals) and induced with IPTG at 100 µM for 18 h at 18°C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM TRIS-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole pH 8.0, 1.4 mM β-mercaptoethanol), and lysed by French Press. The crude lysate was then centrifuged at 27 000 x g for 45 min and was subsequently applied to a 10 mL Ni²⁺-NTA (Qiagen) column. The column was then washed with 100 column volumes of lysis buffer. Polyhistidine-tagged Hsp104 was isolated by elution with lysis buffer containing 200 mM imidazole pH 8.0. Pooled peak fractions were dialyzed overnight against cleavage buffer (50 mM TRIS-HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA pH 8.0, 1.4 mM β-mercaptoethanol) and then digested by TEV protease (Life Technologies) at a protease to Hsp104 ratio of 1:50. After removal of the His-tag, the protein was dialyzed for 2 h against lysis buffer lacking imidazole and subsequently was re-applied onto a 10 mL Ni²⁺-NTA column. The flow through was dialyzed against running buffer (20 mM TRIS-HCl pH 8.0, 50 mM NaCl, 1.4 mM β-mercaptoethanol and 10% glycerol) overnight after which it was further purified by anion exchange chromatography using a 5 mL HiTRAP Q HP column (Amersham) equilibrated in running buffer. The column was then washed with 20 column volumes of running buffer containing 150 mM NaCl. Hsp104 was eluted using a 150-700 mM NaCl gradient. Peak fractions were analyzed by SDS-PAGE, pooled and dialyzed into storage buffer (20 mM
TRIS-HCl pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, 10% (v/v) glycerol) and frozen at -80°C. Recombinant human Hsp70 and yeast Ydj1 were purified as previously described (Freeman et al. 1995; Glover and Lindquist 1998).

2.3.3 Quantitative thermotolerance assays

YPH499Δ104 (MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, hsp104::LEU2) transformed with low copy number vectors for the galactose-inducible expression of Hsp104, were grown to saturation in complete synthetic medium lacking histidine (CSM; Bio101) supplemented with 2% glucose and diluted into the same medium supplemented with 2% galactose, 0.1% glucose for growth to mid-log phase (~2 × 10⁶ cells/mL). After a pre-treatment at 37°C for 1 h, 1 mL of the culture was transferred to pre-warmed sterile glass tubes in a 50°C water bath. Aliquots of cells were removed at various time points and the appropriate dilutions were plated onto rich medium (YPD) and incubated at 30°C for 2-3 days. Survival was calculated as the number of colonies formed by 50°C-treated cultures at each time point compared to those that had received only the pre-treatment.

2.3.4 Western blot analysis of Hsp104 expression

Cells (1 × 10⁸) were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM EDTA pH 8.0) containing protease inhibitors and transferred to 1.5 mL microcentrifuge tubes. Glass beads (0.5 mm; Biospec Products) were added and the cells were broken on a mixer (Model 5432, Eppendorf) operating at 4°C for 45 min. Cell debris was removed by centrifugation at 16,000 × g for 10 min. After separation by SDS-PAGE on 6-20% gels, proteins were transferred to PVDF. Hsp104 was detected with a rabbit polyclonal antibody generated
against recombinant Hsp104. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham) recorded on a Versadoc imaging system (Biorad). Equal loading of protein was ensured by Coomassie blue staining of duplicate SDS-PAGE gels.

2.3.5 Analytical gel filtration

Proteins were analyzed on a 1 × 30 cm Superose 6 column (AP Biotech) in a buffer consisting of 50 mM Tris-HCl pH 8.0, 20 mM NaCl, 10 mM MgCl₂, and 2 mM DTT. The protein content of each 0.4 mL fraction was determined by a dye binding microassay (BioRad) using bovine serum albumin as a standard. To determine the elution profile of unassembled Hsp104 a sample of the wild type protein was chromatographed in the same buffer with 200 rather than 20 mM NaCl.

2.3.6 Refolding assays

Firefly luciferase (FFL; Promega) was denatured in refolding buffer (25 mM HEPES-KOH pH 7.5, 150 mM potassium acetate, 10 mM magnesium acetate, and 10 mM DTT) containing 8 M urea for 30 min at 30°C. The unfolded protein was rapidly dispersed into ice cold refolding buffer, aliquoted into pre-cooled tubes, flash frozen in liquid N₂ and stored at -80°C. Frozen, aggregated FFL was thawed on ice immediately before use. Refolding reactions contained refolding buffer, 5 mM ATP, an ATP regenerating system consisting of 20 mM phosphocreatine and 0.02 units of phosphocreatine kinase, 2.5 µM each of Hsp104, recombinant human Hsp70 and recombinant yeast Ydj1. After 90 min of refolding at 25°C, duplicate 1 µL aliquots were dispersed into luciferase assay reagent (Promega) and light emission was recorded in a luminometer (Lumat LB 9507, EG&G Berthold).
2.3.7 ATPase Assays

All reaction components were equilibrated at 25°C for 4 min prior to the initiation of each reaction by the addition of 25 µL ATP to 2 µg of protein in 25 µL of 20 mM HEPES-KOH, pH 7.5, 20 mM KCl, 10 mM MgCl₂, 2 mM EDTA, 2 mM DTT. After 1 min reactions were stopped by the addition of 100 µL of Malachite green followed after 1 min by 12.5 µL of 34% (w/v) citric acid. Absorbance at 620 nm was determined in a microtitre plate reader (Molecular Devices). All activities were determined through the linear range of ATP hydrolysis and all measurements were corrected for phosphate present in reactions containing ATP alone.

2.3.8 Fluorescence spectroscopy

Hsp104 fluorescence was analyzed as previously described (Hattendorf and Lindquist 2002). All solutions were filtered (0.22 µm) or centrifuged (16 000 × g for 10 min.) to remove particulate matter and all titrations were carried out at 4°C to limit ATP hydrolysis. Proteins were dialyzed against 20 mM HEPES-KOH pH 7.5, 20 mM NaCl, and 10 mM MgCl₂ for 4 h prior to use. Protein concentration was determined by UV absorbance at 276 nm (ε = 31 900 M⁻¹cm⁻¹). Emission was measured at 346 nm at 4°C using a SPEX Fluorolog-3 (Jobin-Yvon), with an excitation wavelength of 295 nm and a 5 nm bandpass. Nucleotide was titrated into a 1.5 µM protein solution from a stock solution (20 mM for ATP, 2 mM for ADP). After each addition the mixture was equilibrated with stirring for 5 min before reading. Buffer-subtracted fluorescence was corrected for dilution by nucleotide addition and the inner filter effect. Data were fitted to a single-site Hill equation for cooperative binding using MacCurveFit. Each curve is the average of three independent titrations.
2.3.9 Fluorescence microscopy

For expression of Hsp104, mutant DNA segments were subcloned into a vector based on pLA28SX104b with an N-terminally fused GFP domain. Cells expressing GFP-Hsp104 fusion proteins were cultured as described for thermotolerance experiments. Mid-log phase cultures were shifted from 25°C to 37°C for 1 h. Live unfixed cells were examined before and after heat shock with a standard epifluorescence microscope (Axioskop 2, Zeiss) equipped with a 100 ×, 1.4 NA objective. Images were recorded with a CCD camera (Spot Jr., Diagnostic Instruments).

2.4 Results

2.4.1 Role of conserved tyrosine residues in Diaphragm 1 and 2 in Hsp104 function in vivo

Members of the Hsp100 family have either two (Class 1) or a single (Class 2) AAA+ module each of which contains a nucleotide binding domain (NBD) and an α-helical small domain (SD). Figure 2.1A compares the sequences of NBD1 and NBD2 of Class 1 Hsp100s, *S. cerevisiae* Hsp104, *T. thermophilus* ClpB and *E. coli* ClpA, as well as the single NBD of Class 2 Hsp100s *E. coli* ClpX and *H. pylori* HslU. In Class 1 Hsp100s, the GYVG loop or Diaphragm 2 (using the nomenclature established for ClpA (Guo et al. 2002), is located in NBD2. In the sequence of Hsp104 the GYVG Tyr is amino acid residue 662. An analogous region, Diaphragm 1 is located in NBD1 of Class 1 Hsp100s. Notably Diaphragm 1 also contains a conserved Tyr residue (Tyr 257 in Hsp104) that we speculated might have a function analogous to the GYVG Tyr in NBD2.
To probe the importance of both Diaphragm 1 and Diaphragm 2 in Hsp104 function we replaced Tyr 257 and Tyr 662 with Ala. Each residue was important for Hsp104 biological function since neither Hsp104\textsuperscript{Y257A} nor Hsp104\textsuperscript{Y662A} provided wild type levels of thermotolerance (Fig 2.1B), although Western blot analysis of cell extracts indicated that Hsp104\textsuperscript{Y257A} and Hsp104\textsuperscript{Y662A} were expressed at the same level as the wild type protein (Fig 2.1C). However, the degree of impairment was significantly different. Survival of Hsp104\textsuperscript{Y662A}-expressing cells, in which the replacement modifies the GYVG motif in NBD2, was no better than cells lacking Hsp104 altogether. In contrast, the survival of Hsp104\textsuperscript{Y257A} was reduced only about 10-fold relative to cells expressing the wild-type protein. We therefore focused further analyses on the GYVG motif and axial channel of NBD2.

To characterize the functional consequences of further amino acid substitutions at Tyr 662, we replaced Tyr with other aromatic amino acids (Y662W, Y662F) and an additional non-conservative substitution (Y662K). We also constructed an Hsp104 variant (Hsp104\textsuperscript{E645K}) analogous to an \textit{At}Hsp101 encoded by the \textit{hot1-1} allele that profoundly impairs the acquisition of thermotolerance in \textit{Arabidopsis thaliana} (Hong and Vierling 2000). Based on the structure of \textit{Tt} ClpB (Lee \textit{et al.} 2003), Glu 645 in Hsp104 is likely situated in the axial channel in the same region as Diaphragm 2. Neither Hsp104\textsuperscript{E645K} nor Hsp104\textsuperscript{Y662K} complemented the thermotolerance defect of YPH449\textsuperscript{Δ104} cells (Fig 2.2A). Survival of cells expressing Hsp104\textsuperscript{Y662F} or Hsp104\textsuperscript{Y662W} was slightly reduced compared to cells expressing Hsp104\textsuperscript{WT}. Western blot analysis indicated that all proteins were expressed at levels comparable to Hsp104\textsuperscript{WT} (Fig 2.2B).
2.4.2 Diaphragm 2 mutants are specifically defective in facilitating protein refolding

To correlate the thermotolerance functions with the measurable biochemical characteristics of Hsp104, purified proteins were tested in refolding reactions alone or supplemented with Hsp70/40 chaperones using denatured, aggregated FFL as the refolding substrate. Refolding by Hsp104^{Y662F} and Hsp104^{Y622W} was comparable to that of Hsp104^{WT} while the refolding activities of Hsp104^{Y662A}, Hsp104^{Y662K} were undetectable (Fig 2.2C). The refolding activity of Hsp104^{E645K} was severely impaired but not completely eliminated. Thus, the ability of each derivative of Hsp104 to refold aggregated protein in vitro correlated well with its ability to confer thermotolerance in vivo.

Many amino acid substitutions in the 2nd AAA\(^+\) module of Hsp104 impair the assembly of Hsp104 (Parsell et al. 1994; Schirmer et al. 2001). Because intersubunit contacts between AAA\(^+\) modules are critical for the constitution of fully functional ATP binding sites, impaired assembly simultaneously impairs ATPase activity of Hsp104. Two lines of evidence indicated that neither of these biochemical properties were significantly altered by the amino acid substitutions in the axial channel that impair thermotolerance or protein refolding. First, all Hsp104 mutants were assembled into hexamers indistinguishable from Hsp104^{WT} when analyzed by gel filtration chromatography (Fig 2.2D). Second, although we detected slight differences in the kinetics of basal ATP hydrolysis among the Hsp104 derivatives (Table 2.2), these differences were neither severe nor correlated with their ability to refold protein or to provide thermotolerance. Thus Hsp104^{E645K}, Hsp104^{Y662A} and Hsp104^{Y662K} represent a
Figure 2.2 The effect of substitutions in the NBD2 axial channel on Hsp104 function. (A) Thermotolerance was determined as described in the legend to Figure 2.1B. (B) Expression levels of Hsp104 proteins were determined as described in the legend to Figure 2.1C. (C) Purified recombinant Hsp104 was used to refold aggregated FFL without (-) and with (+) purified Hsp70 (human) and Hsp40 (yeast Ydj1). Results were normalized to the refolding yield obtained in the complete refolding reaction containing Hsp104WT. (D) Purified recombinant Hsp104 proteins were analyzed by gel filtration in low ionic strength buffer without nucleotide. Dotted line represents the elution profile of Hsp104WT in buffer containing 200 mM KCl in the absence of nucleotide to indicate the elution position of unassembled (monomeric) Hsp104.
Table 2.2 Kinetics of ATPase hydrolysis by Hsp104 and mutants

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<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min•µg)</th>
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<td>WT</td>
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<td>Y662F</td>
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novel type of Hsp104 mutant that is specifically impaired in protein refolding. The data with respect to the properties of Hsp104$^{Y662A}$ are consistent with the observation that HslU, in which the GYVG Tyr (Tyr 91) is replaced with Gly, has normal ATPase activity and assembly properties but is specifically impaired in protein degradation (Song et al. 2000). In a more recent report, the analogous E. coli ClpX Y153A mutant was reported to be likewise specifically impaired in the degradation of proteins (Siddiqui et al. 2004).

To ask whether amino acid substitutions in NBD2 that impair protein refolding might also impair the stable interaction of Hsp104 with its targets in living cells, we examined the localization of GFP-Hsp104 fusion proteins in unstressed and heat-shocked yeast (Fig 2.3). At the level of light microscopy, we have recently shown that Hsp104 is diffusely distributed in unstressed cells but forms a small number of intense cytosolic foci in mildly heat-shocked cells$^2$. This observation is consistent with previous immunoelectron microscopy showing that Hsp104 accumulates at the periphery of electron-dense aggregates in heat shocked cells, but remains diffuse in the absence of aggregates (Fujita et al. 1998). We also demonstrated that derivatives of Hsp104 that do
Figure 2.3 Effect of substitutions in the NBD2 axial channel of Hsp104 on the distribution of Hsp104-GFP in living cells. The subcellular distribution of GFP alone or GFP fused to the indicated Hsp104 mutant was determined by fluorescence microscopy of live cells before (CON) or after a mild heat treatment at 37°C for 1 h (HS).
not oligomerize remain diffuse in heat-shocked cells suggesting that hexamer formation is at least one determinant of a stable interaction between Hsp104 and its thermally aggregated targets in cells (Tkach and Glover, manuscript submitted). Therefore, Hsp104-GFP fusion proteins can be used to test for association of Hsp104 with cellular substrates. For all of the axial channel mutants, the Hsp104-GFP fusion proteins displayed the same pattern of subcellular distribution as the wild type fusion protein. Thus, these results suggest that disabling mutations in the NBD2 axial channel impairs the processing of Hsp104 targets for disaggregation and refolding but is not required for substrate recognition and initial binding.

2.4.3 Tryptophan fluorescence reveals nucleotide-dependent diaphragm movement

Since Hsp104 contains no naturally occurring Trp residues and Hsp104\textsuperscript{Y662W} functions well in thermotolerance and protein refolding, we used Y662W as a fluorescent probe to monitor local changes in the environment of the GYVG loop in response to nucleotide binding. Given the similarity between biochemical and biological properties of Hsp104\textsuperscript{E645K} and Hsp104 with disabling substitutions in the GYVG Tyr residue, we further speculated that impairment of protein refolding in Hsp104\textsuperscript{E645K} might be associated with perturbations in the structure or dynamics of functional elements in the Hsp104 axial channel. We therefore constructed a doubly-substituted Hsp104\textsuperscript{E645K/Y662W} to test this possibility. To distinguish nucleotide-induced conformational changes associated specifically with the axial channel from other, more global effects of nucleotide binding on Hsp104 structure, we used as a control a previously characterized mutant, Hsp104\textsuperscript{Y819W}, in which the unique Trp is situated in small domain of AAA\textsuperscript{+}
module 2 (SD2) of Hsp104 on the exterior of the hexamer and whose fluorescence is influenced solely by nucleotide binding at NBD2 (Hattendorf and Lindquist 2002).

As previously observed (Hattendorf and Lindquist 2002), nucleotide binding enhanced the fluorescence of Hsp104$^\text{Y819W}$ (Fig 2.4A). Dissociation constants (K$_d$) derived from ADP and ATP titration curves corroborated the finding (Hattendorf and Lindquist 2002) that the affinity for ADP binding by NBD2 was about 6-fold higher than for ATP binding (Table 2.3). We also confirmed that Hsp104$^\text{Y819W}$ can be used as a fluorescent probe for nucleotide binding specifically at NBD2. Dissociation constants for ATP and ADP binding using a fluorescent probe introduced at NBD1 (Y257W) were significantly different from those reported for NBD2 (see 3.4.3; Fig 3.3A, Table 3.2). Parallel experiments with the double Hsp104$^{\text{E645K/Y819W}}$ mutant demonstrated that E645K confers a slight increase in the affinity of NBD2 for ATP and a decrease in the affinity for ADP but does not significantly influence the degree of nucleotide-dependent fluorescence changes in Trp 819 at saturating concentrations of nucleotide.

In contrast to Hsp104$^\text{Y819W}$ the fluorescence of Hsp104$^\text{Y662W}$ was quenched on titration with either ATP or ADP (Fig 2.4B). Dissociation constants for ATP and ADP binding were roughly the same for the two different Trp probes suggesting that the local environment of Trp 662, like that of Trp 819, is determined by nucleotide binding to NBD2 (Table 2.3). The Hill coefficients determined by fitting titration curves to a single site Hill equation indicated that the Y662W substitution impaired the cooperativity of ATP but not ADP binding. Most importantly the fluorescence of Trp 662 probe was significantly influenced by the second disabling amino acid substitution, E645K. In the
Figure 2.4 Fluorescence of single Trp mutants of Hsp104. (A) The fluorescence of Hsp104<sup>Y819W</sup> (open symbols) and Hsp104<sup>E645K,Y819W</sup> (filled symbols) was measured at 4°C in the absence or presence of increasing concentrations of ADP or ATP. Each curve is derived from the combined data from three independent titrations. Error bars indicate the standard deviation at each point. Note that the horizontal axes differ by one order of magnitude. (B) Tryptophan fluorescence of Hsp104<sup>Y662W</sup> (open symbols) and Hsp104<sup>Y662W,E645K</sup> (filled symbols) analyzed as in (A). A summary of the kinetic parameters derived from these data is presented in Table 2.3.
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<td>0.9 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
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<tr>
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<tr>
<td>% ΔF</td>
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<td>-10.4 ± 0.7</td>
<td>25 ± 1</td>
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<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt; (µM)</td>
<td>9 ± 2</td>
<td>6 ± 1</td>
<td>10 ± 1</td>
<td>14 ± 1</td>
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<tr>
<td>n</td>
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<td>1.2 ± 0.2</td>
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<td></td>
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<tr>
<td>F/A&lt;sup&gt;c&lt;/sup&gt; (×10&lt;sup&gt;-7&lt;/sup&gt;)</td>
<td>6.79 ± 0.10</td>
<td>7.53 ± 0.69</td>
<td>2.94 ± 0.30</td>
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<sup>a</sup> percent change in fluorescence from nucleotide-free to nucleotide-saturated protein  
<sup>b</sup> n = Hill coefficient  
<sup>c</sup> Fluorescence at 346 nm/absorbance at 280-nm  
All data represent mean of 3 to 4 independent measurements ± standard deviation
presence of E645K, the fluorescence of Trp 662 was higher (less quenched) at saturating concentrations of ATP and lower (more quenched) at saturating concentrations of ADP than in the otherwise wild type protein. Even though E645K specifically influences fluorescence changes in Trp 662 on nucleotide binding we measured the ratio between fluorescence at 346 nm and absorbance at 280 nm to determine if E645K also influenced the fluorescence of the Trp residue in the absence of bound nucleotide. Indeed, E645K increased the fluorescence of both Trp 662 and Trp 819 by about 11% above that of the otherwise wild type protein (Table 2.3) indicating the possibility that E645K perturbs the global structure of AAA+ module 2 Hsp104 in addition to influencing the dynamics of the GYVG loop.

2.5 Discussion

Several lines of experimental evidence have established that Hsp100s that form complexes with proteases unfold their targets and deliver them into the proteolytic chamber by threading the extended polypeptide through the axial channel formed by the Hsp100 oligomer (Ortega et al. 2000; Singh et al. 2000; Ishikawa et al. 2001). We examined the possibility that Hsp104 uses an analogous mechanism during extraction of misfolded proteins from aggregates by examining the role of specific residues in the axial channel of Hsp104 in protein refolding and thermotolerance. Three novel observations from our study support this hypothesis. First, nonconservative substitutions of Tyr in the GYVG motif of NBD2 result in a specific defect in protein refolding, while not substantially affecting other biochemical features of Hsp104 including assembly, ATP hydrolysis and substrate recognition. These observations are consistent with the effects of similar substitutions in the GYVG motifs of ClpX and HslU on ATP-dependent
protein degradation (Song et al. 2000; Siddiqui et al. 2004). Second, using unique Trp residues as fluorescent probes, we find that the local environment of Trp 662 in the GYVG axial channel motif is strongly influenced by nucleotide binding to NBD2, as is seen with the repositioning of the GYVG loop in different nucleotide-bound states of HslU (Wang et al. 2001). Third, a different amino acid substitution, E645K, also specifically inhibits protein refolding and strongly influences fluorescence quenching of Trp 662 in the GYVG loop in response to nucleotide binding at NBD2.

The strongest evidence that positioning of the flexible GYVG loop regulates the aperture of the Hsp100 axial channel comes from crystal structures of HslU (Sousa et al. 2000; Wang et al. 2001). Recently published structures of other Hsp100s including ClpX (Kim and Kim 2003), ClpB (Lee et al. 2003) and ClpA (Guo et al. 2002) reveal many important details related to Hsp100 structure and function even though none were crystallized in the biologically relevant hexameric state. Nonetheless, the GYVG loop of ClpX (Kim and Kim 2003) and the Diaphragm 1 or Diaphragm 2 regions of ClpB were unstructured (Lee et al. 2003) indicating that these segments are indeed flexible. In the ClpA structure, the Diaphragm 1 region was only partially structured, but the GYVG motif was sufficiently rigid to trace entirely (Guo et al. 2002). In the case of Diaphragm 2 of ClpA, stabilization of the flexible region may be due to the presence of a structural magnesium ion in the crystallized protein. It is plausible that this structural magnesium is not a feature of functional ClpA but that its presence artfactually stabilizes and distorts the structure of the ClpA GYVG motif. With this notable exception, the available structural data suggest that Hsp100 NBD domains contain flexible regions that could act in a dynamic and coordinated fashion to attenuate the aperture of the axial channel.
We found that Hsp104\textsuperscript{Y257A} was significantly impaired in thermotolerance but not as dramatically as Hsp104\textsuperscript{Y662A}. In a previous study, using a semi-quantitative assay for thermotolerance, no impairment in the function Hsp104\textsuperscript{Y257W} was detected (Cashikar \textit{et al.} 2002). Therefore the Diaphragm 1 region of Hsp104, like the Diaphragm 2 region, is similarly sensitive to the elimination of the Tyr side chain but functions normally if replaced by other aromatic amino acids. It is now established that Trp 819 (Hattendorf and Lindquist 2002) and Trp 662 (this study) serve as fluorescent probes that are specifically responsive to nucleotide binding to NBD2. We are currently exploring the utility of Trp 257 as a probe for nucleotide binding to Hsp104 NBD1.

Since the function of Hsp104\textsuperscript{Y662A} was more profoundly impaired than Hsp104\textsuperscript{Y257A}, we focused our detailed analysis on the Diaphragm 2 region of Hsp104. We used Trp fluorescence as a means of monitoring dynamic changes in the physical environment of the GYVG motif in response to nucleotide binding. Fluorescence changes in Hsp104\textsuperscript{Y662W} suggest that in the absence of nucleotide, Trp 662 is at least partially shielded from solvent, possibly indicating that the GYVG loop is positioned close to the walls of the axial channel. On either ATP or ADP binding the GYVG apparently moves to a different position that places the Trp residue in a more solvent exposed environment, probably into the aqueous environment of the axial channel. We cannot exclude the possibility that the close proximity of six Trp residues deployed in the axial channel of an Hsp104 hexamer contributes to the fluorescence signal of the Hsp104\textsuperscript{Y662W} mutant. In fact, the slightly impaired thermotolerance and refolding functions and diminished cooperativity of ATP binding to NBD2 we detected in Hsp104\textsuperscript{Y662W} suggest that the substitution of Tyr 662 with somewhat bulkier Trp residues
in the GYVG loop is not completely benign. However, the finding that Hsp104E645K has essentially the same biochemical properties as Hsp104Y662A and Hsp104Y662K including a specific refolding defect, combined with finding that E645K specifically perturbs the nucleotide dependent positioning of the GYVG Trp probe, lends additional support to the idea that the structural integrity of the Hsp104 axial channel and the dynamic nature of the GYVG motif is important for Hsp104 function.

Hsp100s that target proteins for degradation interact with their protease partner complex through specific motifs located in the SD of the AAA− module (AAA+ module 2 in the case of ClpA). This architecture imposes an obligatory directionality to protein translocation that would proceed, in the case of ClpA, from AAA+ module 1 to AAA+ module 2 that contains the GYVG motif (Reid et al. 2001). Assuming that this directionality is a conserved feature of the Hsp100 common mechanism, aggregated substrates would be initially encountered by AAA+ module 1 of Hsp104 and extruded from the hexamer through an exit pore in AAA+ module 2. Since the Hsp104 variants that we studied in detail are impaired by amino acid substitutions associated with exit side of the protein-conducting channel we did not anticipate that substrate recognition by AAA+ module 1 would be impaired. This was borne out by the finding that relocalization of GFP-Hsp104 fusion proteins in response to mild heat shock was essentially the same for each protein irrespective of its ability to subsequently process proteins for refolding in vitro.

Our results do not imply that the functions of the two AAA+ modules in Hsp104 are completely independent. Recently a case has been made for the allosteric regulation of the ATPase activity in Hsp104 NBD1 by nucleotide binding to NBD2 (Hattendorf and
Lindquist 2002) suggesting a means whereby distinct functions of the two NBDs could be coordinated. Based on the observation that ATP hydrolysis at NBD2 is much slower than at NBD1 and that the affinity of NBD2 for ADP is several-fold higher than for ATP, it was proposed that NBD2 remains in the ADP bound state for prolonged periods of time during which the ATPase activity of NBD1 is stimulated. Another intriguing observation is that the rate of ATP hydrolysis at NBD1 is stimulated by the interaction of poly-L-lysine with the Hsp104 C-terminal acidic tail and inhibits the refolding of aggregated FFL (Cashikar et al. 2002). Originally it was proposed that poly-L-lysine mimics the surface of large protein aggregates suggesting that aggregate recognition is a function of the acidic C-terminal tail of Hsp104. However, others find that the poly-L-lysine simultaneously stimulates the ATPase and disaggregation activity of ClpB even though prokaryotic Hsp104 orthologs lack the acidic C-terminus (Strub et al. 2003). Alternatively, we speculate that poly-L-lysine binding to Hsp104 might mimic an interaction between the C-terminal tail of Hsp104 and unfolded polypeptides exiting the axial channel. By this means, translocation intermediates could also act as allosteric effectors of ATPase hydrolysis at NBD1 and thus stimulate the processive unfolding of target proteins or their extraction from aggregates.

Finding that a major refolding pathway for Hsp104-dependent reactions involves critical elements in the axial channel of the protein supports the unfolding/threading or “molecular ratchet” model of Hsp104-mediated disaggregation (Glover and Tkach 2001), but does not exclude the possibility that Hsp104 simultaneously acts as “molecular crowbar” as a means of exposing chaperone binding sites in protein aggregates. However, since we detected no residual protein refolding in reactions containing
Hsp104Y662A or Hsp104Y662K we must assume that these mutants simultaneously impair both processes, or that misfolded proteins or small aggregates released by the crowbar action of Hsp104 perhaps mediated by the coiled-coil domains in AAA+ module 1 (Lee et al. 2003), are further processed for refolding in a manner that depends on the integrity of the axial channel. Intriguingly, two different mutations in the GYVG loop of AtHsp101 were found as intragenic suppressers of a missense mutation in the coiled-coil domain that dominantly inhibits hypocotyl elongation in A. thaliana seedlings at high temperature. In this case, the gain-of-function of AtHsp101 with an altered coiled-coil domain appears to require a functional GYVG loop apparently linking the function of these two structural features.

Future research will focus not only on reconciling the diverse observations derived from structure and function analyses of Hsp104/ClpB itself but will also elucidate how the Hsp104/ClpB-mediated disaggregation is coupled to the Hsp70 chaperone machinery forming a so called “bichaperone network” (Goloubinoff et al. 1999). An attractive feature of the unfolding/threading model of Hsp104 function is that, rather than partially-folded, partially-aggregated proteins expected to be produced by a crowbar mechanism, the end product of the disaggregase would be extended polypeptide chains that are ideal substrates for Hsp70 binding (Rudiger et al. 1997). Although it is conceivable that the disaggregase and refolding components of the bichaperone network function entirely independently, a physical association between the components is an appealing notion. An interaction with T. thermophilus ClpB and DnaK has been recently reported (Schlee et al. 2004). Although the site of DnaK binding was not established in these studies, logic dictates that direct channeling of substrates from ClpB to DnaK
would be rendered most efficient if DnaK were situated near the exit pore in the C-terminal \( \text{AAA}^+ \) domain.

Indeed the yeast homolog of Hop (Hsc70-Hsp90-organizing protein), Sti1, has been found in association with the extreme C-terminal segment of Hsp104 (Abbas-Terki et al. 2001). Even more recently it has been reported that Sti1 also stimulates the ATPase activity of Ssa1 (yeast cytosolic Hsp70) by about 200-fold (Wegele et al. 2003). The significance of these interactions with respect to the function of Hsp104 has yet to be established. However, one exciting possibility is that Sti1, which has two TPR domains that bind acid C-terminal motifs, may interact simultaneously with both Hsp104 and Ssa1. Thus Sti1 could function as a scaffold tethering yeast Ssa/Hsp70s to the C-terminus of Hsp104 where they would be well positioned to interact with extended polypeptides as they are extruded from the disaggregase. Although Sti1 is not obligatory for Hsp104-dependent refolding in an \textit{in vitro} system composed of purified components, it may be important under some conditions for the efficient reactivation of critical thermolabile targets \textit{in vivo}.

2.6 Acknowledgements

The authors wish to thank Dr. Frances Sharom and Dr. David Isenman for helpful discussions, Dr. David Williams for critical reading of the manuscript, and Rachel Long for able technical assistance. R.L. was supported by a Natural Sciences and Engineering Research Council Undergraduate Summer Studentship, J.M.T. by a NSERC Postgraduate Scholarship and JRG by a Canadian Institutes of Health Research scholarship. The work was funded in part by a Premier’s Research Excellence Award to J.R.G. and by a CIHR
operating grant MOP-57863. Work in the laboratory of E.V. was supported by DOE grant DE-FG03-99ER20338.

2.7 Footnotes

1 Abbreviations used: PCR, polymerase chain reaction; GFP, green fluorescent protein; FFL, firefly luciferase; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 J.M. Tkach and J.R. Glover, manuscript submitted

3 U. Lee, C. Wie, M. Escobar, B. Williams, S.-W. Hong, and E. Vierling, manuscript submitted
3 Peptide and protein binding in the axial channel of Hsp104: insights into the mechanism of protein unfolding

Ronnie Lum, Monika Niggemann, and John R. Glover

Monika Niggemann collaborated with me in the construction and screening of the solid phase peptide arrays. I performed all of the other experiments.

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3.1 Abstract

The AAA+ molecular chaperone Hsp104 mediates the extraction of proteins from aggregates by unfolding and threading them through its axial channel in an ATP-driven process. An Hsp104-binding peptide selected from solid phase arrays enhanced the refolding of a firefly luciferase-peptide fusion protein. Analysis of peptide binding using tryptophan fluorescence revealed two distinct binding sites, one in each AAA+ module of Hsp104. As a further indication of the relevance of peptide binding to Hsp104 mechanism, we found that it competes with the binding of a model unfolded protein, reduced carboxymethylated α-lactalbumin. Inactivation of the pore loops in either AAA+ module prevented stable peptide and protein binding. However, when the loop in the first AAA+ was inactivated, stimulation of ATPase turnover in the second AAA+ module of this mutant was abolished. Drawing on these data, we propose a detailed mechanistic model of protein unfolding by Hsp104 in which an initial unstable interaction involving the loop in the first AAA+ module simultaneously promotes penetration of the substrate into the second axial channel binding site and activates ATP turnover in the second AAA+ module.
3.2 Introduction

Hsp104 is a AAA\(^+\) protein disaggregate that functions in yeast in the resolubilization and reactivation of thermally denatured and aggregated proteins (Parsell et al. 1994; Glover and Lindquist 1998). In unstressed cells, Hsp104 is critical to the mitotic stability of the yeast prions \([\text{PSI}^+]\), \([\text{PIN}^+]\), and \([\text{URE3}]\) (Chernoff et al. 1995; Derkatch et al. 1997; Moriyama et al. 2000). Hsp104 and its bacterial ortholog ClpB are members of the Hsp100/Clp family of proteins (Tkach and Glover 2006). Other Hsp100s, such as ClpA, ClpX, and ClpY (HslU), unfold and unidirectionally translocate polypeptides through a central axial channel (Weber-Ban et al. 1999; Ortega et al. 2000; Singh et al. 2000; Ishikawa et al. 2001; Reid et al. 2001). Crystal structures of HslU (Sousa et al. 2000; Wang et al. 2001) and cryoelectron microscopic reconstructions of ClpB (Lee et al. 2007) reveal that the diameter of the axial channel is regulated by flexible loops whose conformation is regulated by the nucleotide status of the nucleotide binding domain of each AAA\(^+\) module. Modification of these loops impairs protein translocation and/or degradation implying that these loops play critical roles in translocation (Siddiqui et al. 2004; Weibezahn et al. 2004; Hinnerwisch et al. 2005; Park et al. 2005). Likewise, mutation of the flexible loops of Hsp104 and ClpB results in refolding defects suggesting that all Hsp100s employ a similar unfolding/threading mechanism to process substrates whether they are ultimately degraded or refolded (Lum et al. 2004; Weibezahn et al. 2004; Kurahashi and Nakamura 2007). Despite the growing body of knowledge regarding the unfolding and translocation mechanism of Hsp104, the determinants of the initial stage of the unfolding process, substrate recognition and binding, remain unclear.
In other Hsp100s, recognition of specific peptide sequences initiates unfolding and translocation. Protein substrates of ClpXP generally contain recognition signals of roughly 10-15 residues that can be located either at the N- or C-terminus (Flynn et al. 2003). The SsrA tag, an 11-amino acid peptide (AANDENYALAA) that is appended to the C-terminus of polypeptides by the action of tmRNA on stalled ribosomes (Gottesman et al. 1998), is a particularly well-studied example of an Hsp100 targeting peptide. The SsrA tag physically interacts with both ClpA and ClpX, targeting the polypeptides for degradation by ClpAP and ClpXP (Haebel et al. 2004). The N-terminal 15-aa peptide of RepA (MNQSFISDILYADIE) is another example of a peptide that when fused either to the N or C terminus of GFP, is sufficient to target the fusion protein for recognition and degradation by ClpAP (Hoskins et al. 2000).

Refolding of proteins trapped in aggregates requires not only Hsp104/ClpB but also a cognate Hsp70/40 chaperone system (Glover and Lindquist 1998; Goloubinoff et al. 1999). Evidence suggests that the Hsp70 system acts prior to the Hsp100, initially to produce lower order aggregates that still lack the ability to refold to the native state (Zietkiewicz et al. 2006). A ClpB mutant containing a substitution in the coiled-coil domain is defective in processing aggregates that are dependent on the DnaK co-chaperone system but has no defect in the processing of unfolded proteins, suggesting a role for the coiled-coil domain in mediating a transfer of substrates from DnaK to ClpB (Haslberger et al. 2007). While it is possible that Hsp70/40 may act as adaptor proteins that present refolding substrates to Hsp104/ClpB, it is not an obligatory pathway. In the absence of Hsp70, Hsp104 alone remodels yeast prion fibres formed by Sup35 and Ure2 (Shorter and Lindquist 2006). Furthermore, Hsp104 in the presence of mixtures of ATP
and slowly hydrolysable ATP analogues or a mutant of Hsp104 with reduced hydrolytic activity in the second AAA\(^+\) module can refold aggregated GFP and activate RepA for DNA-binding without additional chaperones (Doyle et al. 2007). These observations suggest that Hsp104 can directly and independently recognize and process substrates, although the molecular basis of this interaction is unclear.

We hypothesize that, like other Hsp100s, Hsp104 may initially engage substrates by binding to peptides displayed on the surface of aggregated proteins. Using peptide arrays, we found that potent Hsp104-binding peptides were enriched in charged and hydrophobic amino acids. Fusion of an Hsp104-binding peptide to the C-terminus of firefly luciferase (FFL) enhanced its refolding in an Hsp104-dependent refolding reaction. Peptide binding to Hsp104, analyzed by fluorescence quenching using Hsp104 mutants containing tryptophan probes in the first (D1) and second (D2) AAA\(^+\) modules, revealed the existence of at least two unique peptide binding sites. Soluble peptide also competed for binding with a model unfolded protein, reduced carboxy-methylated α-lactalbumin (RCMLa). We also analyzed protein and peptide binding of Hsp104 variants with amino acid substitutions in the axial channel and determined that inactivation of either pore loop abolished peptide and protein binding. However, stimulation of the ATPase activity in D2 was dependent on an intact loop in D1. These data demonstrate the potential for multiple modes of interaction between Hsp104 and segments of unfolded proteins as they are translocated through the axial channel and provide a hypothetical framework for allosteric triggering of ATP hydrolysis in protein unfolding.
3.3 Materials and Methods

3.3.1 Plasmids, DNA manipulation, and recombinant protein expression and purification

3.3.1.1 Hsp104 mutagenesis

All oligonucleotide sequences used are listed in Table 3.1. The E285A and Y257W mutants were constructed by following the Quickchange (Stratagene) method using pBSKII+ containing the EagI/SacI segment of Hsp104 or from Hsp104Y257A (Lum et al. 2004) and primers E285A(A) and E285A(B), and primers Y257W(A) and Y257W(B), respectively. The E687A mutant similarly was constructed using pBSKII+ containing the SalI/Spel segment of Hsp104 using primers E687A(A) and E687A(B). Products were digested with DpnI, transformed into competent E. coli cells, and colonies were screened for the destruction of a StyI-sensitive site for Y257W, the destruction of an NspI-sensitive restriction site for E285A, and the presence of a BtsI-sensitive restriction site for E687A. The expected sequences were confirmed by DNA sequencing. The altered segments were subcloned in place of the corresponding wild type EagI/SacI segment or SalI/Spel segment of pPROEX-HT-b 104. The Hsp104Y257A, Hsp104Y662A and Hsp104Y662W mutants were previously constructed (Lum et al. 2004). The Hsp104Y257A/Y662W mutant was constructed by subcloning a BamHI/SalI fragment from pLA28SX104Y257A into pPROEX-HT-b 104Y662W. Hsp104ΔN and Hsp104ΔN\text{trap} were constructed by amplification of a segment of DNA using pPROEX-HT-b 104 with primers ΔN(FWD) and ΔN(REV). The PCR product was digested with BamHI/EagI and
Table 3.1

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<td>E285A(B)</td>
<td>CCATTACCATTAATTTGAAATGTCATCAATGAAATAACAC</td>
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<tr>
<td>E687A(A)</td>
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<td>E687A(B)</td>
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*Bold indicates codon 285*

*Bold indicates codon 687*

*Bold indicates codon 257*

ligated into pPROEX-HT-b 104 or pPROEX-HT-b 104^nmp^ digested with the same enzymes

### 3.3.1.2 Construction of firefly luciferase-peptide fusion constructs

A segment of DNA was amplified from pPROEX-LUC, containing the WT FFL open reading frame, an N-terminal 6-His tag followed by a Tobacco Etch Virus (TEV) protease cleavage site, and lacking a functional peroxisomal targeting signal, using the LUC(FWD) and LUC(REV) primers and digested with NcoI. The overhanging sequence was filled in using the large subunit of DNA polymerase I (Klenow) followed by BamH1.
digestion and ligation to BamHI-digested pBSKII+. The sequence was confirmed by DNA sequencing. The NcoI/BamHI fragment was then subcloned into p416Gal1 (p416Gal1-LUC) for expression in yeast. Cartridge-purified oligonucleotide pairs encoding 14-mer peptides (p370(A), p370(B), p530(A), p530(B), pSGG(A), and pSGG(B)) at a concentration of 5 nM in 10 mM TRIS-HCl pH 8, 50 mM NaCl, 1 mM EDTA pH 8 were phosphorylated using polynucleotide kinase, annealed by heating to 95°C and slowly cooling to 25°C (∆0.1°C/5sec), and inserted into p416Gal1 LUC digested with BamHI/XhoI. Correct insertion was confirmed by sequencing. For recombinant production of polyhistidine-tagged FFL-fusion proteins, PacI/XhoI segments from p416Gal1-LUC series constructs were subcloned into pPROEX-LUC.

3.3.1.3 Protein purification

All Hsp104 variants were expressed and purified as described elsewhere (Lum et al. 2004). Ydj1 was purified as described previously (Cyr et al. 1992). For purification of recombinant Ssa1, an S. cerevisiae strain (SSA1, ssa2, ssa3, ssa4, pCAUHSEM-SSA1) was grown at 30°C to mid-log phase in YP containing with 2% glucose. The culture was then supplemented with 0.1 volume of 10X YP, 2% glucose, and 100 µM CuSO4, and the cells were allowed to induce overnight. Ssa1 was then purified essentially as described elsewhere (Cyr et al. 1992).

For expression and purification of FFL and mutant variants, plasmids were transformed into BL21Codon plus cells and expression of N-terminal poly-histidine tagged FFL was induced in mid-log phase with 100 µm IPTG at 18°C overnight. Harvested cells were resuspended in lysis buffer (20 mM TRIS pH 8, 400 mM NaCl, 10
mM imidazole, and 1.4 mM β-mercaptoethanol) and lysed by French Press. The crude lysate was then centrifuged at 27 000 x g for 45 min and was subsequently applied to a 10 mL Ni\textsuperscript{2+}-NTA (Qiagen) column. The column was then washed with 100 column volumes of lysis buffer. Polyhistidine-tagged FFL was isolated by elution with lysis buffer containing 200 mM imidazole pH 8.0. The pooled peak fractions were diluted to 2 mg/mL and then dialyzed against running buffer (20 mM TRIS-HCl pH 8.0, 50 mM NaCl, 1.4 mM β-mercaptoethanol, and 10% glycerol) overnight. The dialysate was further purified by anion exchange chromatography using a 5 mL HiTRAP Q HP column (Amersham) equilibrated with the same buffer. Following a 20 column volume wash with running buffer, FFL was eluted using a 150-300 mM NaCl gradient. Peak fractions were dialyzed twice against 50 mM TRIS pH 8, 150 mM NaCl, 1 mM EDTA, 1mM DTT, 0.8 M ammonium sulphate, and 2% glycerol, and frozen at -80°C. Protein concentrations were determined using the Biorad Assay Reagent with BSA as a standard.

3.3.2 Peptide synthesis

Peptides arrays were produced by spot synthesis on cellulose membranes according to the manufacturer’s directions (Intavis, Germany). Soluble peptides were synthesized at the Advanced Protein Technology Center (Hospital for Sick Children, Toronto, Canada). Stock peptide solutions were made freshly by resuspending to 1 mM in sterile water. Concentrations were determined by measuring absorbance at 280 nm or using the Biorad Assay Reagent with BSA as a standard.
3.3.3  **Hsp104 binding to peptide arrays**

Arrays were blocked in 1X Blocking Solution (Sigma Aldrich) diluted in binding buffer (50 mM TRIS HCl pH 8, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT), rinsed three times in binding buffer, and overlayed with 35 nM Hsp104\(^{\text{trap}}\) in the presence of 2 mM ATP for 1 h at room temperature. Unbound Hsp104 was removed by extensive washing in binding buffer containing ATP. Bound protein was then transferred to PVDF using a semidry blotter and Hsp104 was detected with a rabbit polyclonal antibody (see 2.3.4). Immunoreactive spots were detected by enhanced chemiluminescence (Amersham Biosciences) and recorded on a Versadoc imaging system (Bio-Rad). Spot density was determined using IP Lab Gel 2.0. The frequency of amino acid occurrence was calculated as follows:

\[
\text{Observed frequency} = \frac{\text{(no. of aa 'x' in binders)}}{\text{(total no. of aa in binders)}}
\]

\[
\text{Total frequency} = \frac{\text{(no. of aa 'x' in all peptides)}}{\text{(total no. of aa in all peptides)}}
\]

\[
\text{Frequency} = \frac{\text{(Observed Frequency/Total Frequency)}}{1} – 1
\]

A poly-L-lysine spot on each array was used as an internal positive control for Hsp104 binding and as a standard to compare spot intensities between blots.

3.3.4  **Fluorescein labeling of reduced carboxymethylated \(\alpha\)-lactalbumin**

Reduced carboxymethylated \(\alpha\)-lactalbumin (RCMLa; Sigma) labeling with fluorescein isothiocyanate (FITC) (Invitrogen) was performed according to the manufacturer’s directions. The labeled protein (fRCMLa) was purified on a Sephadex G-
25 column (Amersham Biosciences) equilibrated with 20 mM sodium phosphate pH 7.5. Peak fractions were pooled, filtered, and stored at 4°C in the dark until use.

### 3.3.5 Fluorescence spectroscopy

Nucleotide binding measured by changes in Trp fluorescence was performed as previously described (Lum et al. 2004). All solutions were filtered (0.22 µm) or centrifuged (16 000 x g for 10 min. at 4°C) to remove particulate matter. To measure peptide binding, fluorescence of 0.6 µM Hsp104 containing 2 mM nucleotide was measured at 352 nm at 25°C using a Spex Fluorolog-3 (Jobin-Yvon), with an excitation wavelength of 295 nm and a 5 nm bandpass. Peptides were titrated from a 100 µM stock solution. Each sample was stirred for 5 min before reading. Data were fitted to a single-site saturation equation for binding using MacCurveFit.

Fluorescence anisotropy was measured as previously described (Bosl et al. 2005) in reaction buffer (20 mM HEPES KOH pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1.4 mM β-mercaptoethanol) with several exceptions. 0.6 µM Hsp104trap was incubated with or without 2 mM nucleotide at 25°C for 5 min. For inhibition of fRCMLa binding to Hsp104, competitors were added to a solution containing Hsp104 and ATP, incubated for 10 min, and reactions were initiated by the addition of fRCMLa to 0.06 µM. The fraction of fRMCLa bound to Hsp104 was calculated using the equation:

\[
% \text{Bound} = 100 \times \frac{(r-r_{\text{free}})}{[(r_{\text{bound}}-r) + (r-r_{\text{free}})]}
\]
where “r” represents anisotropy. For competition of fRCMLa binding post-Hsp104-fRCMLa complex formation, fRCMLa was added to initiate the binding reaction and upon completion of the reaction, competitors were added to 9 μM.

### 3.3.6 Refolding of denatured aggregated luciferase

_In vivo_ and _in vitro_ refolding of firefly luciferase (FFL) was performed as described elsewhere (Tkach and Glover 2004). _In vitro_ refolding reactions were supplemented with 100 μM soluble peptides.

### 3.3.7 Luciferase aggregation assay

Experiments were performed as described elsewhere (Lu and Cyr 1998) with several modifications. FFL was thermally aggregated at 0.2 μM in a polystyrene 96-well flat-bottom plate (Sarstedt, Germany) at 42°C in reaction buffer supplemented with 5 mM ATP in the presence or absence of 0.8 μM Ssa1 and 1.6 μM Ydj1. Rates of FFL aggregation were determined by monitoring increases in light scattering using a SpectraMax 340PC® microplate reader (Molecular Devices) at 370 nm.

### 3.3.8 ATPase activity

A coupled enzymatic spectrophotometric assay in combination with an ATP-regenerating system (Norby 1988) was used to monitor ATP hydrolysis by Hsp104. All reagents were purchased from Sigma Aldrich unless otherwise indicated. Reactions were carried out in reaction buffer containing 3 mM phosphoenolpyruvate, 0.23 mM NADH (Bioshop, Canada), 70 units/ml pyruvate kinase, and 100 units/ml L-lactate dehydrogenase (both obtained from rabbit muscle), 2 mM ATP, and 0.2 μM Hsp104. Assays were performed in a polystyrene 96-well flat-bottom plate using a SpectraMax
340PC microplate reader (Molecular Devices) at 30°C monitoring NADH oxidation at 340 nm. ATPase rate was calculated from the slope $\frac{dA_{340\text{ nm}}}{dt}$ using a molar extinction coefficient for NADH of $\varepsilon_{340\text{ nm}} = 6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$. Data were fitted to either a line or a rectangular hyperbola.

3.4 Results

3.4.1 Screen for Hsp104-interacting peptides

We initiated our search for Hsp104-interacting peptides by screening solid-phase arrays of peptides corresponding to overlapping 13-mer segments of a variety of proteins. Array membranes were incubated with an Hsp104 ‘trap’ mutant (E285A/E687A, Hsp104\textsuperscript{trap}; see Fig 3.1A for a schematic guide to Hsp104 domains and residues relevant to this work) that binds but does not hydrolyze ATP (Weibezahn et al. 2003). After electrophoretic transfer of bound proteins, Hsp104 was detected with a polyclonal antibody. Strong Hsp104 binding peptides were defined as peptides in the 95th percentile by normalized spot intensity (156 out of 3908 peptides). The relative fractional occurrence of each amino acid in the strongest binders against the natural occurrence of all 20 amino acids in all peptides was determined (Fig 3.1B). We found that Hsp104-binding peptides were enriched in the aromatic residues phenylalanine and tyrosine, the charged residues lysine and aspartic acid, and the polar residue asparagine. Serine, glycine, proline, and tryptophan were under-represented in these peptides. The abundances of cysteine and methionine residues on the arrays were too low to be considered statistically significant.

Molecular chaperones are thought to be able to discriminate between folded and unfolded proteins by the high degree of exposure of hydrophobic residues on the surface
Figure 3.1  Hsp104 binding to peptide arrays. (A) The primary sequence elements of Hsp104. NTD, N-terminal domain; D1, AAA’1 module; CCD, coiled-coil domain; D2, AAA’2 module; CTD, C-terminal domain; A, Walker A; B, Walker B. (B) Frequency of amino acid occurrence in strong Hsp104-binding peptides. (C) Raw luminescence data from a 13-mer peptide array derived from the S. cerevisiae Sup35 GTPase domain. Amino acid position of the starting peptide in each row is indicated on the left. Asterisk (*) indicates the end of the Sup35 sequence. (D) Ribbon diagram of homology model of the GTPase domain of S. cerevisiae Sup35 created by Swiss-Model (Schwede et al. 2003) and based on the crystal structure of S. pombe Sup35 (1R5B) (Kong et al. 2004). Hsp104-binding peptides are coloured by accessibility on a linear gradient (Yellow = accessible, Blue = buried) using Swiss-Pdb viewer (Guex and Peitsch 1997) and are space-filled. The numbers correspond to amino acid number in Fig 1C. Dagger (†) indicates that the structure has been rotated 180° about the vertical axis.
of misfolded proteins compared to their native conformers. To provide insight into the location of Hsp104-binding peptides within a natively folded protein, we used binding data from a peptide array corresponding to the primary sequence of the globular domain of *S. cerevisiae* Sup35 (Fig 3.1C), and mapped them onto a model based on the crystal structure of the *Schizosaccharomyces pombe* protein (Kong *et al.* 2004). Analysis of the solvent accessibility of these peptides indicated that they were generally buried in the interior of the folded protein (Fig 3.1C) consistent with their generally high content of hydrophobic amino acid residues. However, because further studies on peptide-binding to Hsp104 in solution would be dependent on the solubility of peptides over a broad range of concentrations, we focused on those array peptides containing hydrophobic amino acids intermixed with charged or polar residues.

### 3.4.2 Peptides can enhance refolding of aggregated protein

Other Hsp100s apparently initiate unfolding by binding to specific peptide sequences. For example, the SsrA tag appended onto the C-terminus of GFP is sufficient to direct the degradation of GFP by the ClpXP protease (Kim *et al.* 2000). However, peptides selected for their ClpX binding properties from arrays conferred ClpX binding to a GFP peptide fusion protein but failed to promote GFP degradation in the presence of ClpP (Thibault *et al.* 2006). This result could represent the manifestation of the formal possibility that some peptides on arrays could interact with the probe protein in an adventitious manner. For example, peptides could bind to the outer surfaces of the chaperone as opposed to within the axial channel where substrate processing most likely occurs.
We therefore adopted a functional approach to test whether candidate peptides could enhance the refolding of aggregated FFL, a robust model refolding substrate for Hsp104 \textit{in vivo} (Singer and Lindquist 1998; Tkach and Glover 2004) and \textit{in vitro} (Glover and Lindquist 1998). In preliminary experiments candidate peptides were fused to FFL as C-terminal extensions and expressed in yeast. None of the peptides that failed to bind Hsp104 on solid phase arrays and that were incorporated into these experiments as negative controls, influenced FFL-peptide fusion protein refolding following thermal denaturation. However, some but not all peptides that were judged to be strong Hsp104-binders on solid phase arrays enhanced the recovery of thermally denatured FFL \textit{in vivo} (data not shown).

To more rigorously determine the influence of peptide extensions on FFL refolding, two peptides that both bound Hsp104 on arrays and enhanced \textit{in vivo} refolding of FFL, p370 (KLSFDDVFEREYA) and p530 (NDFQEQQEQAPE), as well as a non-binding control peptide pSGG (SGGSGGSGGSGGS), were further tested in \textit{in vitro} refolding reactions using Hsp104 along with the Hsp70/40 chaperones Ssa1 and Ydj1 (Glover and Lindquist 1998). FFL-pSGG was refolded with the same efficiency as FFL lacking a peptide extension (Fig 3.2A). Fusion of p530 to FFL modestly enhanced the refolding yield while FFL-p370 was refolded completely. These results are consistent with the notion that Hsp104-binding peptides confer an additional element that enhances the recognition or processing of FFL that is not present in FFL lacking a peptide extension.

However, it is possible that enhanced refolding of FFL-peptide fusions could be attributable to differences in the aggregation characteristics or in the ability of fusion
Figure 3.2 Hsp104-dependent refolding and interaction with aggregated recombinant FFL-peptide fusion proteins. (A) Urea-denatured and aggregated FFL-variants were incubated with Hsp104, Ssa1, and Ydj1 at 30°C and refolding was monitored. Error bars indicate the standard deviation of three independent experiments. (B) FFL-variants were thermally aggregated at 42°C in the absence (black, -) or presence (grey, +) of Ssa1 and Ydj1. Turbidity at 370 nm was monitored.
proteins to interact with Hsp70/Hsp40 chaperones. To test this, FFL and the extended variants were heat denatured under conditions where aggregation, measured by light scattering, was partially suppressed by the Hsp70/Hsp40 in the presence of ATP (Lu and Cyr 1998). The aggregation of FFL and FFL-p370 in the absence of chaperones and the degree of aggregation suppression in the presence of Hsp70/40 were not different (Fig 3.2B). Addition of p530 and pSGG as C-terminal extensions on FFL modestly improved the Hsp70/40-dependent suppression of aggregation. However, because these differences did not correlate with enhanced refolding from the aggregated state, we conclude that peptide-mediated enhancement of refolding by peptide extension is primarily Hsp104-dependent.

### 3.4.3 Distinct peptide binding sites in the first and second AAA⁺ modules

The axial channel of Hsp100s (Wang et al. 2001; Lee et al. 2007) feature flexible loops that govern the aperture of the pore. The position of these loops within the central channel of the Hsp104 hexamer is controlled by nucleotide binding and previously we exploited this property to measure nucleotide binding to D2 in a mutant Hsp104 containing a unique Trp substitution for a conserved Tyr residue on the $^{661}\text{GYVG}^{664}$ D2 loop (Lum et al. 2004). In this work, we extended these measurements using Hsp104$^{Y257W}$ containing an analogous Trp residue on the $^{256}\text{KYKG}^{259}$ D1 loop. Fluorescence of the Y257W probe was quenched in response to nucleotide binding (Fig 3.3A). Dissociation constants for ATP and ADP binding to D1 (Table 3.2) were significantly higher than those previously reported for D2 (see 2.4.3; Fig 2.4, Table 2.3) indicating that indeed Hsp104$^{Y257W}$ and Hsp104$^{Y662W}$ independently measure nucleotide
Table 3.2
Summary of nucleotide-dependent fluorescence changes in single Trp-Hsp104
All data represent the mean of three independent trials ± SD

<table>
<thead>
<tr>
<th></th>
<th>Y257W</th>
<th>Y662Wb</th>
<th>Y819Wc</th>
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</thead>
<tbody>
<tr>
<td>ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ ($\mu$M)</td>
<td>170 ± 60</td>
<td>60 ± 2</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>$\Delta F$ (1-$F/F_o$)$^a$</td>
<td>0.106 ± 0.003</td>
<td>0.21 ± 0.02</td>
<td>n.d.</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ ($\mu$M)</td>
<td>500 ± 150</td>
<td>9 ± 2</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>$\Delta F$ (1-$F/F_o$)$^a$</td>
<td>0.137 ± 0.007</td>
<td>0.077 ± 0.009</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$ Percent change in fluorescence from nucleotide-free ($F_o$) to nucleotide-saturated protein

$^b$ Adapted from Lum et al, 2004.

$^c$ Adapted from Hattendorf et al, 2002.

n.d. indicates not determined

binding to each module. The lower affinity for nucleotide binding at D1 is consistent with the relatively high $K_m$ observed for ATP hydrolysis by D1 (Hattendorf and Lindquist 2002).

The flexible loops in the Hsp100 axial channel appear to play a role in binding substrates and in their subsequent processing by translocation through the axial channel (Siddiqui et al. 2004; Weibezahn et al. 2004; Hinnerwisch et al. 2005; Park et al. 2005). We hypothesized that peptide binding may also influence the conformation of residues in the axial channel of Hsp104 and therefore applied the site-specific probes to investigate peptide binding to Hsp104. The fluorescence of Hsp104$^{Y257W}$ in D1 in the presence of ADP or AMP-PNP, a non-hydrolyzable ATP analogue, was quenched upon titration with p370 (Fig 3.3B). Titration of the non-binding control peptide pSGG did not significantly alter the fluorescence of Hsp104$^{Y257W}$. Calculated dissociation constants (Table 3.3)
**Figure 3.3 Peptide binding to NBD1 and NBD2.**

(A) Fluorescence of single Trp mutant Hsp104^Y257W titrated with increasing concentrations of ADP (left) or ATP (right). Each curve is derived from the combined data from three independent titrations. Error bars indicate the standard deviation at each point. Peptide binding to (B) Hsp104^Y257W and (C) Hsp104^Y662W was measured with 2 mM AMP-PNP (left) or ADP (right), and increasing concentrations of p370 (filled circles) or pSGG (open circles). Excitation and emission monochromators were set to 295 nm and 352 nm, respectively. Each data point is the mean of three independent experiments and error bars indicate the standard deviation. Data were fitted to an equation for single-site saturated binding.
Table 3.3

Summary of fluorescence changes upon titration of p370 in single Trp-Hsp104
All data represent the mean of three independent trials ± SD

<table>
<thead>
<tr>
<th></th>
<th>Y257W</th>
<th>Y662W</th>
<th>E645K/Y662W</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMP-PNP (2 mM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>$84 \pm 1$</td>
<td>$300 \pm 100$</td>
<td>$190 \pm 30$</td>
</tr>
<tr>
<td>$\Delta F$ ($1-F/F_0$)(^a)</td>
<td>$0.106 \pm 0.003$</td>
<td>$0.116 \pm 0.006$</td>
<td>$0.133 \pm 0.005$</td>
</tr>
<tr>
<td><strong>ADP (2 mM)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>$96 \pm 3$</td>
<td>$160 \pm 20$</td>
<td>not detected</td>
</tr>
<tr>
<td>$\Delta F$ ($1-F/F_0$)(^a)</td>
<td>$0.137 \pm 0.007$</td>
<td>$0.060 \pm 0.002$</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Percent change in fluorescence from peptide-free ($F_o$) to peptide-saturated protein

indicated that p370 binds with roughly the same affinity to D1 irrespective of the nucleotide bound.

Parallel experiments with Hsp104\(^{Y662W}\) indicated that titration of p370 into AMP-PNP or ADP-bound Hsp104 also quenched Trp fluorescence when the probe is incorporated into the D2 loop (Fig 3.3C). No change in fluorescence was observed when Hsp104\(^{Y662W}\) was titrated with pSGG in either nucleotide-bound state. The binding affinity of p370 to D2 was higher in the ADP-bound state when compared to the AMP-PNP-bound state. The distinct binding affinity for p370 to D1 compared to D2 suggests the existence of at least two peptide binding sites.

Surprisingly, even though p530 binds to Hsp104 on arrays and enhances refolding of FFL \textit{in vivo} and \textit{in vitro}, titration of p530 into solutions containing either Hsp104\(^{Y257W}\) or Hsp104\(^{Y662W}\) with AMP-PNP or ADP did not significantly change the fluorescence intensity of the probes (data not shown). This finding does not exclude the possibility that p530 is capable of binding to Hsp104 at a site that does not influence the
fluorescence properties of the probes used in this analysis and thereby enhances the refolding of a FFL-p530 fusion protein.

3.4.4 Peptide competition for unfolded protein binding to Hsp104

We hypothesized that if p370 binding represents a mechanistically relevant interaction with Hsp104, it should also compete for one or more binding sites used by unfolded proteins. To test this idea, soluble p370, p530, and pSGG were used as competitors for Hsp104 binding to RCMLa, an unfolded and soluble monomeric protein that has been used as a model for chaperone binding (Cyr et al. 1992; Hayer-Hartl et al. 1994; Scholz et al. 1997; Kern et al. 2003) including Hsp104 where RCMLa-binding is ATP-dependent (Bosl et al. 2005). Preincubation of Hsp104trap and ATP with 2 µM p370 resulted in the inhibition of approximately half the binding of Hsp104 to fluorescein-labeled RCMLa (fRCMLa) (Fig 3.4A). Preincubation with p530 at the same concentration had no inhibitory effect. An addition of 5 µM p370 completely abolished fRCMLa whereas at the same concentration the control peptide, pSGG, had no effect. Preincubation of Hsp104trap-ATP with varying concentrations of p370 and fitting the resulting curve with an equation describing a single-site competition reported an IC50 of 2.1 µM (Fig 3.4B).

Although it was previously reported that binding of fRCMLa to ATP-bound Hsp104trap was irreversible (Bosl et al. 2005), we found that under our conditions, incubation of unlabeled RCMLa with preformed complexes resulted in exchange over time (Fig 3.4C). Addition of p370 could also disrupt preformed fRCMLa–Hsp104trap complexes with a time course that nearly overlaps with that of RCMLa. Fitting these
Figure 3.4  **p370 competition with fRCMLa for binding to Hsp104.** (A) Hsp104\textsuperscript{trap} was preincubated with ATP for 10 min. Subsequently, peptides at various concentrations were added and incubated for 5 min. fRCMLa binding was initiated by the addition of fRCMLa. Fluorescence anisotropy was measured with monochromators set to 494 nm (excitation) and 515 nm (emission). Experiments were performed in triplicate and one representative data set is shown. (B) The experiment was performed as described in (A). p370 inhibited Hsp104\textsuperscript{trap} with an IC\textsubscript{50} of 2.1 ± 0.3 µM. Error bars indicate the standard deviation of three measurements. (C) Unlabelled RCMLa (grey circles), pSGG (empty diamonds), or p370 (filled diamonds) were added after Hsp104\textsuperscript{trap}-fRCMLa-ATP complex formation and the change in anisotropy was monitored. Data were fitted to an equation describing a three-component exponential decay process. (D) Refolding of denatured aggregated firefly luciferase by purified recombinant Hsp104, Ssa1, and Ydj1 in the absence or presence of peptide. Results were normalized to the refolding yield obtained in a refolding reaction in the absence of soluble peptide. Error bars indicate the standard deviation of three independent measurements.
curves to equations describing three-component exponential decay could distinguish a rapid exchange component with a half life (t_{1/2}) of 1.2 min, a slower one with a t_{1/2} of 12 min, and a very slow component with a t_{1/2} of 20 h for RCMLa competition, and somewhat faster exchange by p370 with calculated t_{1/2}s of 1.3 min, 8.3 min., and 12 h.

Together, these results indicate that at least one peptide, p370, binds to Hsp104 and competes with fRCMLa for binding. We next asked if p370 could inhibit a refolding reaction where ATP is constantly being hydrolyzed and exchanged. Addition of p370 or pSGG to high concentrations, even as high as 100 µM, did not reduce the yield of refolded FFL (Fig 3.4D), suggesting that p370 may be readily displaced under conditions that permit turnover of ATP. Alternatively, p370 may be strongly out-competed for binding during the processing of protein extracted from aggregates.

3.4.5 The N-terminal domain is dispensable for protein/peptide binding to Hsp104

We next set out to determine where in the Hsp104 molecule competition between protein and peptide binding occurs. To investigate the role of the Hsp104 N-terminal domain (NTD) we constructed an Hsp104 mutant lacking amino acids 1-152 (Hsp104ΔN). This truncation mutant was fully functional in vivo and in vitro in refolding FFL (Fig 3.5A and B). Although the NTD of Hsp104 is dispensable for thermotolerance and prion maintenance in yeast (Hung and Masison 2006), some evidence suggests that the NTD of ClpB modulates its interaction with protein aggregates (Liu et al. 2002; Tanaka et al. 2004; Barnett et al. 2005). To examine the role of the NTD in peptide and protein binding, we constructed a mutant that combined the N-terminal deletion with the Walker B E285A/E687A substitutions (Hsp104ΔN_{trap}). Hsp104ΔN_{trap} in the ATP-bound
Figure 3.5 The N-terminal domain of Hsp104 is dispensable for protein and peptide binding. (A) In vivo refolding of aggregated FFL. Cells were cultured in galactose to induce the expression of Hsp104 and FFL. Log phase cells were heated to 44 °C for 20 min to inactivate FFL. The recovery of FFL activity was normalized to the activity measured in each culture immediately before heat shock. One representative data set is shown. (B) In vitro refolding of denatured aggregated firefly luciferase by purified recombinant Hsp104 without (-) and with (+) purified Ssa1 and Ydj1. Results were normalized to the refolding yield obtained in a complete refolding reaction containing wild type Hsp104. Error bars indicate the standard deviation of three independent measurements. (C) Hsp104\textsuperscript{trap} (square) and Hsp104\textsuperscript{ΔN\textsuperscript{trap}} (diamond) were incubated with fRCMLa and the reaction was initiated by the addition of ATP (filled) or ADP (empty). Fluorescence anisotropy was measured as described in Fig 4A. Experiments were performed in triplicate and one representative data set is shown. (D) Inhibition of fRCMLa binding to Hsp104\textsuperscript{ΔN\textsuperscript{trap}} by preincubation with peptides. The IC\textsubscript{50} for p370 inhibition was 1.3 ± 0.05 µM.
form was able to bind fRCMLa and this binding closely approximated that of the full length protein (Fig 3.5C). p370 inhibited fRCMLa binding to Hsp104ΔNtrap with an IC$_{50}$ of 1.3 µM while excess pSGG had no inhibitory effect (Fig 3.5D). Because the salient features of protein binding and peptide competition are preserved in the absence of the Hsp104 NTD, this indicates that this domain is dispensable for the formation of stable substrate/chaperone complexes.

3.4.6 Peptide stimulation of ATP hydrolysis

Recently, work by others has demonstrated that RCMLa strongly stimulates Hsp104 ATPase activity specifically in D2 (Schaupp et al. 2007). Given this observation, we next investigated whether p370 could substitute for unfolded protein in this stimulation. We constructed Hsp104 variants with Walker B substitutions that prevent ATP hydrolysis in Hsp104 Trap (E285A or E687A) but separately in order to establish the ATPase activity of D1 and D2 independently. Consistent with previous observations with RCMLa, only the Walker B mutation in D2 abolished the stimulation of ATPase activity by p370 (Fig 3.6A) indicating that p370 stimulates ATP hydrolysis in D2. As expected, addition of p530 or the non-binder pSGG to either mutant did not stimulate ATP hydrolysis (data not shown).

Since, in this work, we infer the existence of p370 binding sites in both D1 and D2 (Fig 3.3B and C), we next asked if an intact D2 loop is required to trigger the ATPase activity of D2. Surprisingly, the stimulation of Hsp104$^{Y662A}$ by p370 was not significantly different from that of the wild type protein while Hsp104$^{Y257A}$ was barely responsive to the addition of peptide (Fig 3.6C). The profound defect in Hsp104$^{Y257A}$ stimulation was also observed in the presence of RCMLa (Fig 3.6B). Taken together,
Figure 3.6 Stimulation of ATP hydrolysis by peptide and protein. ATPase activity was measured at 30°C in a reaction containing Hsp104, ATP, and an ATP regeneration system in the presence of p370 or RCMLa. ATPase fold stimulation was normalized to the rate of ATP hydrolysis in the absence of peptide or protein. Each data point is the mean of three independent experiments and error bars represent standard deviations. Data were fitted linearly or to a rectangular hyperbola. Stimulation of ATP hydrolysis of (A) Hsp104E285A (filled) and Hsp104E687A (open) by p370, and of Hsp104WT (square), Hsp104Y257A (filled circle), and Hsp104Y662A (open circle) by (B) RCMLa and (C) p370.
these data suggest that an intact D1 loop in Hsp104 is required to activate ATP hydrolysis in D2 and that the D2 loop has no significant role in this stimulation.

3.4.7 Perturbation of the axial channel alters protein and peptide binding

Since an intact D1 loop is required for protein and peptide stimulation of Hsp104 ATPase activity while the D2 loop is dispensable, we predicted that only the D1 loop is critical for protein and peptide binding. Surprisingly, binding of fRCMLa to Hsp104<sup>Y257A</sup> or Hsp104<sup>Y662A</sup> in the presence of ATPγS was not detected suggesting that stable fRCMLa binding requires both loops (Fig 3.7A).

Because we inferred the existence of two p370 binding sites in D1 and D2, we speculated that in contrast to protein binding, peptide binding to D1 and D2 might occur independently. To test this we constructed a mutant of Hsp104 with the D1 loop inactivated by the Y257A substitution and incorporating the Trp probe into the D2 loop (Hsp104<sup>Y257A/Y662W</sup>). We also constructed the inverse mutant with the D2 loop inactivated and the Trp probe incorporated into the D1 loop (Hsp104<sup>Y257W/Y662A</sup>). No changes in fluorescence of Hsp104<sup>Y257A/Y662W</sup> or Hsp104<sup>Y257W/Y662A</sup> with either AMPPNP or ADP were observed upon titration with p370 (Fig 3.7B). Together, these observations suggest that stable protein and peptide binding requires intact D1 and D2 loops and that these loops function in an interdependent manner.

We previously observed that a non-conservative substitution in the D2 loop (Y662A) abolished Hsp104-mediated thermotolerance whereas the analogous substitution in the D1 loop (Y257A) exhibited an intermediate loss of function (see 2.4.1; Fig 2.1). Given our observation that the D1 loop is critical for stable protein and peptide
Figure 3.7 Perturbation of the axial channel alters protein and peptide binding. (A) Binding of fRCMLa to Hsp104<sup>WT</sup>, Hsp104<sup>Y257A</sup>, and Hsp104<sup>Y662A</sup>. Hsp104 was incubated with fRCMLa and binding was initiated by the addition of ATPγS. Experiments were performed in triplicate and one representative data set is shown. (B) Fluorescence quenching of Hsp104<sup>Y257A/Y662W</sup> (left), and Hsp104<sup>Y257W/Y662A</sup> (right), in response to p370 titration was monitored in the presence of AMP-PNP (closed circle) or ADP (open circle). Each data point is the mean of three independent experiments and error bars indicate standard deviations. (C) Refolding of denatured aggregated firefly luciferase by purified recombinant Hsp104 as described in Fig 5B.
binding, we re-tested the activity of Hsp104\textsuperscript{Y257A} in an \textit{in vitro} refolding assay. Consistent with the protein and peptide binding data, we found that the refolding activity of Hsp104\textsuperscript{Y257A} is severely impaired \textit{in vitro} and only slightly more active than Hsp104\textsuperscript{Y662A} (Fig 3.7C).

3.5 Discussion

Binding of Hsp104 to solid phase peptides supports the hypothesis that Hsp104 distinguishes misfolded proteins from their correctly folded conformers based on the exposure of hydrophobic amino acid side chains. First, the composition of Hsp104-binding peptides is enriched in certain hydrophobic residues including Phe, Tyr, and Leu. Second, when the positions of Hsp104-interacting peptides from the globular domain of Sup35 are mapped onto a three-dimensional model of the domain, the peptides that display Hsp104 binding correspond to polypeptide segments that are only solvent exposed at their ends in the folded protein. While the exposure of these polypeptide segments in denatured conformers may be important for the ability of Hsp104 to discriminate between native and non-native protein complexes, for practical reasons the poor solubility of hydrophobic peptides limits their utility for exploration of the peptide-binding properties of Hsp104. In preliminary trials, hydrophobic peptides solubilized by poly-ionic tags (Cunningham and Deber 2007) also strongly stimulate the ATPase activity of Hsp104 (R.L. and J.R.G., unpublished observation).

Nonetheless, soluble peptides that include hydrophobic as well as charged and polar amino acids appear to be appropriate substrate mimics in most respects. The enhanced refolding of the FFL-p370 fusion protein suggests that the p370 moiety provides an additional determinant that is not present in FFL lacking the extension and
which promotes FFL extraction from aggregates and unfolding by Hsp104. Furthermore, p370 as a soluble peptide recapitulates the properties of an unfolded protein in that it competes for binding of the model unfolded protein RCMLa and displays a similar ability to stimulate the D2 ATPase activity of Hsp104. Neither unfolded protein binding nor the ability of peptide to compete, is dependent on the N-terminal domain of Hsp104 suggesting that these interactions occur primarily in the axial channel formed by the AAA⁺ modules of Hsp104.

A common feature of chaperones is the cycling between high and low affinity states for substrate binding based on conformational changes driven by ATP hydrolysis. In other Hsp100s including ClpA (Wickner et al. 1994), ClpX (Wawrzynow et al. 1995), and ClpB (Weibezahn et al. 2003; Lee et al. 2007), the ATP-bound chaperone undergoes stable substrate binding. This is consistent with the formation of a stable RCMLa/Hsp104 complex with ATP or an ATP analogue bound but not ADP (this work and (Bosl et al. 2005). Based on these observations we anticipated that, in parallel to the ATP-dependent formation of a stable unfolded protein/Hsp104 complex, peptide binding in D1 or D2 or both would exhibit a high affinity state with ATP bound and that in the ADP-bound state the affinity of peptide binding sites would be either greatly diminished or eliminated. In contrast we saw either no change in peptide binding affinity at D1 or even an increase in affinity at the D2 binding site between the AMPPNP and ADP states. We do not know at the present time whether this anomaly is a specific characteristic of p370 or a general feature of peptide binding that is distinct from protein binding.
3.5.1 A model of the Hsp104 reaction cycle

Based on our own observations and those of others, we propose a model for protein unfolding and translocation by Hsp104 consisting of four distinct states (Fig 3.8): the idling state, in which Hsp104 is poised to interact with incoming substrate; a primed state, in which ATPase activity is stimulated by an initial unstable interaction with a polypeptide at D1; a processing state, in which both D1 and D2 participate in binding and translocation; and a prerelease state, in which the polypeptide has traversed the axial channel at D1.

3.5.1.1 The idling state

We define an Hsp104 molecule not engaged by polypeptide and hydrolyzing ATP at a basal rate to be in an idling state. In the absence of ligand, ATP hydrolysis at D1 is relatively slow at ~ 20 min\(^{-1}\) (Hattendorf and Lindquist 2002) while hydrolysis at D2 is barely detectable. The low affinity of D1 for ADP (Fig 3.3A) suggests that this domain is predominantly ATP-bound in the idling state. D2 is likely in the ADP-bound state because of its 10-fold higher affinity for ADP over ATP (see 2.4.3; Fig 2.4, Table 2.3). This characteristic may support the initial interaction with substrate and is consistent with the observation that RCMLa binding is not observed when Hsp104 is in the ADP-bound state (Bosl et al. 2005; Schaupp et al. 2007).

3.5.1.2 The primed state

In other Hsp100s, substrates are translocated along the axial channel and extruded into the chamber of an associated protease for degradation (Hoskins et al. 2000; Kim et al. 2000; Ortega et al. 2000; Singh et al. 2000; Reid et al. 2001; Weibezahn et al. 2004). Indeed, an Hsp104 mutant that interacts with ClpP is capable of translocating
Figure 3.8 A model of Hsp104-mediated unfolding and translocation. The substrate unfolding and translocation mechanism of Hsp104 consists of four distinct stages. In the idling state ATP is slowly turned over in D1 and hydrolytic activity at D2 is essentially quiescent. Upon polypeptide interaction with D1 in the primed complex, ATP hydrolysis at D2 is allosterically enhanced. Conversion of ATP to ADP at D2 in turn stimulates ATP hydrolysis at D1. The reversibility of this interaction indicates that it is unstable. Slowing of hydrolysis at D1 by the inclusion of slowly hydrolysable ATP analogue may enhance the formation of the primed complex. If a segment of polypeptide is sufficiently long to span the distance separating the D1 and D2 loops, the substrate becomes stably associated in the processing complex. The partial remodeling of aggregated proteins by Hsp70/40 chaperones may be required to generate extended polypeptide segments capable of efficiently forming the processing complex. In the prerelease complex the translocating polypeptide is released from D1 returning D2, and in turn, D1 to a less active state similar to the idling state but with last segment of the polypeptide associated with D2. The polypeptide is either spontaneously released or is ejected from Hsp104 by the formation of a new primed complex. See discussion for additional detail.
substrates into ClpP suggesting a directional mechanism for substrate binding and processing along the channel from D1 to D2 (Tessarz et al. 2008). An initial interaction with the D1 loop is consistent with experiments in which a ClpB-binding peptide can be crosslinked to the D1 loop of ClpB (Schlieker et al. 2004). In our experiments, stable protein and peptide binding required both D1 and D2 loops while the activation of ATP hydrolysis at D2 required only an intact D1 loop. In our model, we call this initial D1 loop-dependent interaction the “primed” state. Previous work has suggested that ADP binding to D2 activates hydrolysis at D1 (Hattendorf and Lindquist 2002) and it is reasonable to propose that in the primed state, rapid conversion of ATP to ADP at D2 will result in simultaneous activation of ATP hydrolysis at D1.

Under standard conditions for Hsp104 dependent refolding, it is possible that the Hsp70/40 chaperones act as the rate-limiting step. It has been recently suggested that while the action of Hsp70/40 on aggregates may not efficiently release free polypeptides, it can displace polypeptide segments from the surface of aggregates (Zietkiewicz et al. 2006) and these may act at the formation of the primed state by presenting polypeptide segments in partially disaggregated proteins. When Hsp104 dependent refolding occurs under conditions that do not require Hsp70/40 (Doyle et al. 2007), we propose that diminishing the hydrolysis of ATP at some NBDs using mixtures of ATP and ATPγS or slowing of ATP hydrolysis at D2 by mutation, may promote the formation of the primed state by prolonging a transient state in the idling complex which potentiates substrate interaction.
3.5.1.3 The processing state

Activation of ATP hydrolysis in the primed state serves to capture a substrate at D1 driving it deeper into the axial channel. Because stable binding of RCMLa was abolished in the D2 loop mutant Hsp104Y662A, we propose that only when a substrate encounters the D2 loop, does it become stably associated with Hsp104 and that the interdependent action of D1 and D2 are required for full translocation. The slow formation of a stable RCMLa-Hsp104 complex (about 10 min) under conditions that prevent ATP hydrolysis may reflect the time required for a segment of RCMLa to reach the peptide binding site(s) present at D2 through spontaneous oscillation in the channel rather than a process facilitated by ATP hydrolysis-driven motion of the D1 loop. Using the *T. thermophilus* ClpB crystal structure (Lee et al. 2003) as a model we estimate the distance between the D1 and D2 loops to be approximately 45Å. Hsp70/40, in addition to promoting the primed state, could, by the same mechanism of partial unfolding of aggregates to expose polypeptide loops or termini, facilitate the formation of the processing state as well and may explain in part why binding of aggregates but not monomeric unfolded proteins to ATP-bound ClpB requires DnaK, DnaJ, and GrpE (Haslberger et al. 2007).

As long as there is contact between a substrate and the binding site(s) in D1, the reciprocal allosteric stimulation of ATP hydrolysis in both D1 and D2 will be maintained thus committing the processing complex to rapid unfolding and translocation of the substrate. The ability of Hsp104 to load substrate into ClpP suggests that at least some substrates are fully translocated (Tessarz et al. 2008). However, recent evidence obtained with ClpB demonstrated efficient refolding of protein fusions of misfolded and native
domains without the unfolding of the folded domain, indicating that full translocation is not obligatory (Haslberger et al. 2008). Furthermore, ClpB hexamers are dynamic complexes and exchange subunits on a rapid timescale suggesting that hexamer disassembly may facilitate dissociation of ClpB from very stable aggregates after partial translocation thereby rescuing ClpB from substrate “traps” (Haslberger et al. 2008; Werbeck et al. 2008).

3.5.1.4 The prerelease state

Prior to the final release of substrate from the Hsp104 axial channel, the last segment of translocating polypeptide will be associated only with D2 in a complex that we define as the prerelease state. In this state, allosteric stimulation of hydrolysis at D2 by substrate binding at D1 will also cease and D2 will likely return to its idling ADP-bound state. None of our experiments directly addressed how substrates might be released from the prerelease complex. Since a stable complex likely requires simultaneous interaction with both D1 and D2, it is also likely that a polypeptide, interacting with only D2 is released spontaneously. However, our model predicts that the formation of a hybrid state in which D1 interacts with an incoming substrate polypeptide will result in the restimulation of ATP turnover at D2 and thereby trigger efficient ejection of the previous substrate from D2.

While proteins can be fully threaded through the axial channel of Hsp104, model substrates that are unable to completely traverse the axial channel because they are fused to a stably-folded domain that can not be unfolded by ClpB, are nonetheless, released and refolded (Haslberger et al. 2008). Subunit exchange experiments indicate that ClpB
disassembles and reassembles under processing conditions suggesting an alternative mode of substrate release.

### 3.5.1.5 Structural models of Hsp104

The crystal structure of the Hsp104 hexamer has yet to be determined. However, the structure of the bacterial ortholog ClpB (monomeric) has been solved and used to reconstruct a model of the native hexamer. The reconstructed hexamer describes ClpB as two-tiered, in which the two AAA\(^+\) modules in each monomer sit on top of one another. Additionally, the coiled-coil domains emerge from D1 and are displayed on the exterior of the hexamer (Lee et al. 2003). These features are corroborated by reconstructions of cryoEM images of ClpB (Lee et al. 2007). Notably, a narrow channel penetrates the central axis of the ClpB hexamer. This channel is a common feature of all Hsp100s for which crystal structures are available (Bochtler et al. 2000; Sousa et al. 2000; Wang et al. 2001; Guo et al. 2002; Kim and Kim 2003). While this work was in progress, a cryoEM study of ATP\(_\gamma\)S-bound Hsp104 (Wendler et al. 2007) revealed a strikingly different picture of Hsp104 structure. In this model, Hsp104 forms a large central cavity up to 78Å in diameter capped by the Hsp104 N-terminal domains and with the coiled-coil arms intercalating between adjacent subunits where they form part of the walls of the central cavity and disrupt the domain interactions that are typical of all other AAA\(^+\) proteins. As this model lacks the narrow axial channel that is present in other Hsp100s, it is challenging to interpret our data in terms of the role of axial loop residues in protein or peptide binding. Additional structural and biochemical data is required to explore and corroborate the exceptional features of this model.
3.6 Acknowledgments

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4 A kinetic analysis of protein and peptide binding to Hsp104

Ronnie Lum and John R. Glover

I performed all of the experiments.
4.1 Abstract

The AAA\(^+\) molecular chaperone Hsp104 functions to resolubilize protein aggregates and is a crucial thermotolerance factor in yeast. Hsp104 mediates the extraction of proteins from aggregates by unfolding and threading them through its axial channel in an ATP-driven process. Here, we explore the recognition elements within a model Hsp104-binding peptide that are required for rapid and efficient binding to Hsp104. We found that removal of bulky hydrophobic residues and lysines abrogated the ability of this peptide to: (i) bind directly to Hsp104, (ii) to stimulate ATP turnover by Hsp104, and (iii) to compete for binding with a model unfolded protein to Hsp104. Furthermore, rapid binding of a model unfolded protein to Hsp104 requires an intact N-terminal domain (NTD) and ATP binding at the first AAA\(^+\) module. These results are consistent with a model of protein remodeling by Hsp104 in which an initial substrate binding event requires an exposed hydrophobic peptide segment from an aggregate and that this interaction is stabilized by both the NTD and ATP binding at the first AAA\(^+\) module.
4.2 Introduction

Hsp104 is a member of the Hsp100/Clp subfamily of AAA\(^+\) ATPases (Neuwald et al. 1999). Hsp104 has three known biological functions. First, in thermally stressed cells, Hsp104 is a protein disaggregase that resolubilizes and reactivates thermally denatured and aggregated proteins (Parsell et al. 1994; Glover and Lindquist 1998) Second, Hsp104 is required for the asymmetric distribution of oxidatively damaged proteins during mitosis and plays a role in life-span determination (Erjavec et al. 2007; Tessarz et al. 2009; Liu et al. 2010). Finally, Hsp104 is critical to the mitotic stability of the yeast prions \([PSI^+], [PIN^+], \) and \([URE3]\) (Chernoff et al. 1995; Derkatch et al. 1997; Moriyama et al. 2000).

Other Hsp100s, such as ClpA, ClpX, and ClpY (HslU), unfold and unidirectionally translocate polypeptides through a central axial channel (Weber-Ban et al. 1999; Ortega et al. 2000; Singh et al. 2000; Ishikawa et al. 2001; Reid et al. 2001). Crystal structures of HslU (Sousa et al. 2000; Wang et al. 2001) and cryoelectron microscopic reconstructions of ClpB (Lee et al. 2007) reveal that the diameter of the axial channel is regulated by flexible loops containing a conserved GYVG motif. The conformation of these loops is controlled by the nucleotide status of the nucleotide binding domain of each AAA\(^+\) module (Wang et al. 2001; Lee et al. 2007). Upon hydrolysis of ATP, these loops may sweep downward driving the translocation of a polypeptide toward the axial channel exit before repositioning for the next stroke by nucleotide exchange. In many Hsp100s, including Hsp104, modification of these loops impairs protein translocation implying that these loops play critical roles in the function of Hsp100s (Lum et al. 2004; Siddiqui et al. 2004; Weibezahn et al. 2004; Hinnerwisch et al. 2005; Park et al. 2005; Kurahashi and Nakamura 2007).
Hsp100s cycle between high and low affinity substrate binding states that depend on the nucleotide-bound state of the Hsp100. Interactions between model substrates and Hsp104 are detectable in the presence of ATP and ATP$_\gamma$S, but not in the absence of nucleotide or in the presence of ADP (Glover and Lindquist 1998; Bosl et al. 2005) suggesting that Hsp104 has a binding and release cycle controlled by nucleotide hydrolysis and exchange. Furthermore, the binding of unfolded protein stimulates the ATP hydrolytic activity of the normally quiescent second AAA$^+$ (D2) module of Hsp104 and stabilizes the binding of ATP to the first AAA$^+$ (D1) module (Schaupp et al. 2007). Stimulation requires an intact flexible axial channel loop at D1 as amino acid substitutions in the axial channel flexible loop that prevent protein binding to Hsp104 also prevent ATPase stimulation (Lum et al. 2008). Taken together, an allosteric model was proposed whereby initial binding of a polypeptide segment to the axial channel loop at D1 acts as an allosteric switch that accelerates the ATPase activity of D2 (Lum et al. 2008).

Refolding of proteins trapped in aggregates requires not only Hsp104/ClpB but also a cognate Hsp70/40 chaperone system acting in a concerted manner (Glover and Lindquist 1998; Goloubinoff et al. 1999). Although the involvement of these chaperones in the disaggregation process is well established, the way they associate with protein aggregates remains as yet poorly understood. Several findings indicate that the DnaK system is required in the early stages of protein disaggregation to either extract polypeptides from aggregates (Schlieker et al. 2004) or to remodel the aggregate surface (Zietkiewicz et al. 2006). It is therefore possible that the Hsp70/40 may act as adaptor proteins that present refolding substrates to Hsp104. Following the action of the Hsp70 system, translocation of the polypeptide chain through the central channel of Hsp104 is an important part of the disaggregation process (Weibezahn et al. 2004). The Hsp70 system might also be involved downstream of Hsp104 action, interacting with the substrate
protein being processed by Hsp104, and therefore preventing its reassociation with the aggregate (Weibezahn et al. 2004).

Nevertheless, Hsp104 must bind directly to substrates during the protein remodeling process to initiate unfolding and translocation. In the absence of the Hsp70/40 co-chaperones, Hsp104 can still remodel yeast prion fibres (Shorter and Lindquist 2006) and protein aggregates in vitro (Doyle et al. 2007) suggesting that Hsp104 can directly and independently recognize and process substrates. Hsp104 may distinguish misfolded proteins from their correctly folded conformers based on the exposure of hydrophobic amino acid side chains. Hsp104-binding peptides identified by screens of solid phase peptide arrays were enriched in certain hydrophobic residues, including Phe, Tyr, and Leu (Lum et al. 2008). Furthermore, a soluble peptide composed of a mixture of aromatic, hydrophobic and polar or charged amino acids can recapitulate the properties of an unfolded protein in that it competes for binding of a model unfolded protein and displays a similar ability to stimulate the D2 ATPase activity of Hsp104. These observations lend support to a model in which exposure of hydrophobic peptide segments on the surface of misfolded proteins serve as a targeting signals for Hsp104-mediate protein remodeling.

Although surface hydrophobicity is a likely property that allows Hsp104 to distinguish between aggregates of misfolded proteins and assemblies of natively folded proteins, a detailed biochemical analysis of the specificity of this interaction has not yet been done. In the present study, we have analyzed the relative contribution of hydrophobic side chains to a model peptide–Hsp104 interaction. We found that a reduction in the aromaticity of a model Hsp104-binding peptide correlated with reduced ability to compete for binding of a model unfolded protein, to stimulate the ATPase activity of Hsp104, and to bind directly to Hsp104. Binding to Hsp104,
analyzed by surface plasmon resonance (SPR), revealed that reduced aromaticity also abrogated fast binding ($k_{on}$). Having established that SPR could be used to monitor peptide binding to Hsp104, we next applied this approach to examine protein binding. We found that both an intact N-terminal domain (NTD) and ATP-binding at D1 were required for rapid binding of a model unfolded protein, reduced carboxymethylated $\alpha$-lactalbumin (RCMLa). These results are consistent with an initial substrate binding event that is characterized by the binding of an exposed hydrophobic peptide segment from an aggregate that is stabilized by both the NTD and ATP binding at D1.

4.3 Materials and Methods

4.3.1 Hsp104 mutagenesis

The Hsp104$^{E285A}$, Hsp104$^{E687A}$, Hsp104$^{E285A/E687A}$, and Hsp104$^{\Delta N}$ mutants were constructed as described previously (Lum et al. 2008).

4.3.2 Protein purification

All Hsp104 variants were expressed and purified as described elsewhere (Lum et al. 2004). RCMLa (Sigma Aldrich, USA) was resuspended in sterile water at 10 mg/mL. fRCMLa was prepared as previously described (Lum et al. 2008). Protein concentrations were determined using the Biorad Assay Reagent with BSA as a standard.

4.3.3 Peptide synthesis

Peptides were synthesized on TentaGel S-RAM resin (Rapp Polymere GmbH, Germany) at a 5 µmol scale according to the manufacturer's directions on a MultiPep Synthesizer (Intavis, Germany). The expected mass of the synthesized product was confirmed by mass spectrometry. The purity of each peptide was assessed using HPLC by applying peptides diluted at 0.2 mM in
0.1% TFA onto a C18 reverse phase column. Peptides were eluted using a 0-50% acetonitrile gradient (containing 0.1% TFA) and monitoring absorbance at 215 nm. For biochemical assays, stock peptide solutions were made freshly by resuspending in sterile water. The concentrations of stock solutions were determined by measuring absorbance at 280 nm or using a Micro BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA) with bovine serum albumin as a standard.

4.3.4 Fluorescence anisotropy

Fluorescence anisotropy was measured as previously described (Lum et al. 2008) in reaction buffer (RB, 20 mm HEPES KOH, pH 7.5, 150 mm NaCl, 10 mm MgCl₂, and 1.4 mm β-mercaptoethanol) with several exceptions. Hsp104trap (2 μm) was incubated in 2 mM ATP at 25°C for 5 min. For inhibition of fluorescein-labeled RCMLa (fRCMLa) binding to Hsp104, competitor peptides were added at a final concentration of 10 μM to a solution containing Hsp104 and ATP and incubated for 30 min prior to the initiation of binding by the addition of fRCMLa to 0.2 μM. Peptide concentrations were chosen such that the addition of p370 as a positive control would displace approximately 40% of the fRCMLa from binding to Hsp104. All of the other peptides were added at these same concentrations so that the efficacy of a mutant peptide could be estimated relative to p370. Assays were performed in 96-well black clear flat-bottom non-binding polystyrene plates. Anisotropy was measured on a BMG PHERAstar (BMG LABTECH GmbH, Germany) with excitation and emission filters for 490 nm and 520 nm, respectively.

4.3.5 ATPase assays

ATP hydrolysis was measured essentially as described elsewhere (Lum et al. 2008). Peptides were added to 10 μM and preincubated for 10 min before the addition of ATP. Peptide
concentrations were chosen such that the addition of p370 as a positive control would stimulate the ATPase of Hsp104 by 1.4 fold. All of the other peptides were added at these same concentrations so that the efficacy of a mutant peptide could be estimated relative to p370.

4.3.6 Surface plasmon resonance

Surface plasmon resonance experiments were performed on a Biacore X (Biacore, Piscataway, NJ) instrument at 25°C. Procedures used to couple peptides to the sensor surface were adapted from a protocol described elsewhere (Jones et al. 2004). Briefly, 10 resonance units (RU) of peptide were immobilized on a dextran-coated CM-5 sensor chip (Biacore). For all coupling steps, a running buffer of 20 mM HEPES KOH pH 7.5 at 5 µL/min was used. Chips were made amine reactive by passing a solution of N-hydroxysuccinimide (NHS; Sigma Aldrich) and N-ethyl-N’-(dimethyl-aminopropyl)-carbodiimide (EDC, Sigma Aldrich) over the chip surface. The NHS and EDC were dissolved in ultrapure water to concentrations of 0.5 M and 0.4 M, respectively. A solution of 0.05 M NHS, 0.2 M EDC, and 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 5 buffer were mixed to create the activation solution. Flow cell 1 (FC1) and flow cell 2 (FC2) were activated with a 14 min pulse with the NHS/EDC solution. A 6-carbon spacer was introduced by binding 6-aminohexanoic acid (Sigma Aldrich) at 50 mg/mL in running buffer. The surface was then blocked with 1 M ethanolamine hydrochloride pH 8.5 (Biacore). The carboxylic acid groups of the spacer molecules were activated with a 14 min pulse of freshly diluted NHS/EDC solution. The reactive groups were then converted to primary amines by injecting a 1 M ethylene diamine solution diluted in running buffer for 10 min. The surface was blocked with a 5 min pulse with 1 M ethanolamine hydrochloride pH 8.5. The primary amines were made thiol-reactive by injecting a solution of sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Pierce) dissolved to 20 mM in running buffer for 10 min. Peptides with C-terminal Cys residues were diluted to 0.2 M in
running buffer, reduced by passage through a spin column packed with an immobilized TCEP-disulphide reducing gel, and then coupled to the free maleimide groups on the surface of the chip with a 10 min injection. Finally, the surface was blocked by a 5 min pulse of 1 M β-mercaptoethanol. A reference channel on FC1 was made by coupling a non-binding Hsp104 peptide, pSGG, using the same procedure.

To couple RCMLa to the surface of a CM5 chip, a standard amine coupling protocol was used. FC1 and FC2 were activated by a 14 min pulse with the NHS/EDC solution. FC2 was then injected with a 2 mg/mL solution of RCMLa (Sigma Aldrich) diluted in 0.1 M MES pH 5 for 10 min. 100 resonance units were coupled. A reference channel on FC1 was made by sham activation with NHS/EDC and inactivation with ethanolamine.

To monitor analyte binding, wild-type or mutant Hsp104 was injected at a flow rate of 20 µl/min for 300 s at the concentrations indicated in Figures 4.2 and 4.3, followed by a 90 s buffer flow before initiating the washing phase. The running buffer used was 20 mM HEPES KOH pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1.4 mM β-mercaptoethanol, and 2 mM ATP. The sensor chip was regenerated between successive analyte injections with a 60-s pulse of 6 M guanidine hydrochloride, which brought the sensorgram signal back to baseline. Sensorgram data was analyzed using BiaEvaluation v. 4.1 (Biacore) and kinetic parameters were determined by global fitting of the association and dissociation phases.
4.4 Results

4.4.1 Hydrophobic and charged segments in peptides are crucial for the substrate mimetic attributes of Hsp104-binding peptides

Given that Hsp104-binding peptides are enriched in aromatic residues (phenylalanine and tyrosine) (Lum et al. 2008), we began our analysis of the contribution of hydrophobic side chains to the binding of peptides to Hsp104 by synthesizing mutant peptides of a model Hsp104-binding peptide with varying aromaticity. The peptide chosen was p370 (KLSFDDVFEREYAC) which has previously been shown to mimic the properties of an unfolded protein in that it competes for binding to fRCMLa, and stimulates the ATPase activity of Hsp104. Previously, we observed that Hsp104 has a propensity to bind to peptides enriched in bulky hydrophobic (phenylalanine, tyrosine) and charged (lysine, aspartic acid) amino acids (see 3.4.1; Fig 3.1). We therefore predicted that substitutions of these residues with an amino acid that does not contribute to binding to Hsp104 would then abrogate these properties. To test this hypothesis, we synthesized peptides that contained substitutions of the bulky hydrophobic amino acids with alanine (Table 4.1; F4A, F8A, Y12A, F4A/F8A, F4A/F8A/Y12A) or a charge residue with a small polar amino acid (K1S). To investigate the contribution of charge in the binding of peptides to Hsp104, we synthesized a series of peptides in which charged amino acids were substituted with residues of the opposite charge (D5K/D6K, D5K/D6K/E9K/E11K, K1D).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>pSGG</td>
<td>SSGGGSGGGSGGC</td>
</tr>
<tr>
<td>p370</td>
<td>KLSFDDVFEREYAC</td>
</tr>
<tr>
<td>F4A</td>
<td>KLSADBVFEREYAC</td>
</tr>
<tr>
<td>F8A</td>
<td>KLSFDDAEREYAC</td>
</tr>
<tr>
<td>Y12A</td>
<td>KLSFDDVAEREYAC</td>
</tr>
<tr>
<td>F4A/F8A</td>
<td>KLSADBDAEYAC</td>
</tr>
<tr>
<td>F4A/F8A/Y12A</td>
<td>KLSADBDAEYAC</td>
</tr>
<tr>
<td>D5K/D6K</td>
<td>KLSFKEKVFEREYAC</td>
</tr>
<tr>
<td>D5K/D6K/E9K/E11K</td>
<td>KLSFKEKVFKRKYAC</td>
</tr>
<tr>
<td>K1D</td>
<td>DLSFDDVFEREYAC</td>
</tr>
<tr>
<td>K1S</td>
<td>SLSFDDVFEREYAC</td>
</tr>
</tbody>
</table>
Figure 4.1 Stimulation of ATP hydrolysis and competition with fRCMLa for binding to Hsp104. (top) ATPase activity was measured at 30°C in a reaction containing Hsp104, ATP, and an ATP regeneration system in the presence of p370 or RCMLa. ATPase fold stimulation was normalized to the rate of ATP hydrolysis in the absence of peptide or protein. Each data point is the mean of three independent experiments and error bars represent one standard deviation. (bottom) Hsp104trap was preincubated with ATP for 10 min. Subsequently, peptides at various concentrations were added and incubated for 5 min. fRCMLa binding was initiated by the addition of fRCMLa. After 30 minutes, fluorescence anisotropy was measured with monochromators set to 490 nm (excitation) and 520 nm (emission). Binding was plotted as 1 minus the fraction of fRCMLa bound to Hsp104 in the absence of an inhibitor peptide. Error bars represent one standard deviation of three independent measurements.
We found that removal of a single bulky hydrophobic amino acid had a drastic impact on the efficacy of the peptide in its ability to stimulate ATP hydrolysis by Hsp104 and to compete for binding with a model substrate to Hsp104 (Fig. 4.1, F4A, F8A, Y12A). Removal of two or more bulky hydrophobic residues (F4A/F8A, F4A/F8A/Y12A) rendered the peptide inert and was therefore indistinguishable from a known peptide that does not interact with Hsp104 (pSGG) (Lum et al. 2008).

We next predicted that peptides with reduced ability to stimulate the ATPase activity of Hsp104 and compete for the binding of a model unfolded protein would also exhibit reduced affinity in binding directly to Hsp104. To test this, peptide ligands were coupled to a solid surface and Hsp104 binding was determined by SPR. These experiments employed an Hsp104 “trap” mutant (E285A/E687A, Hsp104\textsuperscript{trap}) that binds but does not hydrolyze ATP and therefore stably binds substrate (Bosl et al. 2005). Hsp104 bound to p370 with a $K_d$ of 104 nM (Fig 4.2, Table 4.2), an affinity that correlates with our previous observation that p370 binds to D1 with a higher affinity than to D2 (see 3.4.3; Fig 3.3, Table 3.3). Substitution of a single Phe with Ala reduced the affinity of the peptide by almost 20-fold. Furthermore, substitution of two Phe residues with Ala resulted in a peptide that bound to Hsp104 with an even higher $K_d$ (Table 4.2). These observations demonstrate that aromaticity is a crucial element of peptide-Hsp104 interactions.

Table 4.2

| Kinetic parameters of ATP-Hsp104\textsuperscript{trap} binding to surface-coupled peptides |
|----------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                       | p370            | D5K/D6K         | F4A             | F8A             | F4A/F8A         |
| $K_d$ (µM)                            | 0.104 ± 0.006   | 0.060 ± 0.004   | 1.63 ± 0.01     | 2.0 ± 0.6       | 2.7 ± 0.3       |
| $k_{on}$ (1/µs)                       | 4.40 ± 0.05 x 10^4 | 6.2 ± 0.3 x 10^4 | 7 ± 1 x 10^3    | 1.3 ± 0.2 x 10^3 | 5 ± 2 x 10^3    |
| $k_{off}$ (1/µs)                      | 4.6 ± 0.2 x 10^3 | 3.5 ± 0.4 x 10^3 | 1.2 ± 0.2 x 10^2 | 2.7 ± 1 x 10^2  | 1.5 ± 0.5 x 10^2 |
Figure 4.2: Sensorgram overlay for the binding of Hsp104\textsuperscript{trap} to surface-coupled p370 assessed by surface plasmon resonance. Hsp104\textsuperscript{trap} was injected onto the surface of a p370-coupled CM5 chip at increasing concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µM, bottom to top). Solutions were injected for 300 s at 20 µL/min. Experiments were performed in triplicate and one representative data set is shown.
As charged residues are also enriched in Hsp104-binding peptides (Lum et al. 2008), we next asked whether these amino acids contribute to the binding properties of p370. Substitution of either the aspartic acid residues or both the aspartic and glutamic acid residues in p370 (D5K/D6K, D5K/D6K/E9K/E11K) with lysine resulted in peptides that, when compared to p370, more efficiently competed with fRCMLa for binding and stimulated the ATPase of Hsp104. SPR analysis of Hsp104 binding to p370^{D5K/D6K} indicated that enhanced fRCMLa competition and ATPase stimulation correlated with an increase in affinity for direct binding to Hsp104 (Table 4.2). Furthermore, removal of the single lysine residue (K1D or K1S) diminished the ability of the peptide to stimulate the ATPase activity of Hsp104 and had only a modest effect on inhibiting fRCMLa binding (Fig 4.1). Surprisingly, substitution of this lysine with aspartic acid (K1D) had a negative impact on the efficacy of the peptide, even though Asp was previously found to be enriched in Hsp104 binding peptides. This suggests that an acidic amino acid cannot sufficiently substitute for lysine in mediating the binding of a peptide to Hsp104. Taken together, these observations suggest that charged residues in addition to bulky hydrophobic amino acids are crucial for direct binding of peptides and are critical determinants of substrate recognition by Hsp104.

4.4.2 A high affinity protein binding state requires the NTD and ATP-bound specifically at D1

We next set out to determine the structural elements in Hsp104 that are required for the efficient binding of substrates. Given that we successfully established SPR as a technique to monitor binding of Hsp104 to peptides, we therefore extended this approach to examine the kinetics of protein binding to Hsp104 using RCMLa as a model substrate. ATP-Hsp104\textsuperscript{trap} bound to RCMLa with high affinity but significantly more noise was observed in the signal when compared to sensorgrams obtained for peptide binding to Hsp104 (Fig 4.3, Table 4.3). This is
Figure 4.3: Sensorgram overlay for the binding of Hsp104 to surface-coupled RCMLa assessed by surface plasmon resonance. Hsp104<sup>trap</sup> was injected onto the surface of a p370-coupled CM5 chip at increasing concentrations (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 µM, bottom to top). Solutions were injected for 300 s at 20 µL/min. Experiments were performed in triplicate and one representative data set is shown.
likely due to the fewer number of RCMLa molecules coupled to the sensor surface when compared to the number of peptides that were covalently attached. We then investigated the role of the Hsp104 N-terminal domain (NTD) in protein binding. Although the NTD is not required for function \textit{in vivo} and \textit{in vitro} in refolding (Lum \textit{et al.} 2008) or for thermotolerance and prion maintenance in yeast (Hung and Masison 2006), some evidence suggests that the NTD of ClpB modulates its interaction with protein aggregates (Liu \textit{et al.} 2002; Tanaka \textit{et al.} 2004; Barnett \textit{et al.}). To examine the role of the NTD in protein binding, we used a previously characterized Hsp104 truncation mutant which lacks amino acids 1–152 (Hsp104ΔN) and contains Walker B E285A/E687A substitutions (Hsp104ΔN\textsuperscript{trap}; see 3.4.5, Fig 3.5C) (Lum \textit{et al.} 2008). Hsp104ΔN\textsuperscript{trap} in the ATP-bound form was able to bind RCMLa, although with significantly weaker affinity than Hsp104\textsuperscript{trap} (Table 4.3). Furthermore, the rate at which Hsp104ΔN\textsuperscript{trap} bound to RCMLa (k_{on}) was an order of magnitude slower than Hsp104\textsuperscript{trap}, indicating a role for the NTD in stabilizing the acquisition of the substrate-bound state.

<table>
<thead>
<tr>
<th>Table 4.3</th>
<th>Kinetic parameters of ATP-bound Hsp104 binding to surface-coupled RCMLa</th>
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<tbody>
<tr>
<td></td>
<td>Hsp104\textsuperscript{trap}</td>
</tr>
<tr>
<td>K_d (µM)</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>k_{on} (1/Ms)</td>
<td>4.0 ± 0.6 x 10^4</td>
</tr>
<tr>
<td>k_{off} (1/s)</td>
<td>0.009 ± 0.003</td>
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We previously presented a model that described an initial substrate binding event as requiring ATP bound to the D1 of Hsp104 (Lum \textit{et al.} 2008). Given our observation that rapid binding of RCMLa requires an intact NTD, we next investigated whether ATP binding at D1 is also required for the fast binding of substrates. We used Hsp104 variants with Walker B substitutions that prevent ATP hydrolysis in Hsp104\textsuperscript{trap} (E285A or E687A) but separately to establish the roles of ATP binding at D1 and D2 independently. The E285A substitution that ensures that the D1 domain is ATP-bound has comparable affinity to the fully ATP-bound
Hsp104trap (Table 4.3). In contrast, when D1 is free to hydrolyze ATP while D2 remains in the ATP-bound state, we observed a significantly reduced affinity for RCMLa.

4.5 Discussion

4.5.1 Hydrophobic and charged amino acids as recognition elements for Hsp104

A hallmark of all molecular chaperones is binding promiscuously to misfolded proteins and generally not to their native conformers. For example, several extensive analyses of Hsp70 binding to peptide libraries reveal that Hsp70 proteins recognize short hydrophobic polypeptide stretches that are in an extended conformation (Flynn et al. 1991; Blond-Elguindi et al. 1993; Gragerov and Gottesman 1994; Rudiger et al. 1997). These binding motifs are abundant in protein sequences. However, in the native state these sites are generally buried in the hydrophobic core of the protein, thereby explaining the promiscuous binding of Hsp70 to unfolded polypeptides. Recognition of exposed hydrophobic side chains was similarly observed in many other molecular chaperones such as the Hsp100s ClpB (Schlieker et al. 2004) and ClpA (Thibault et al. 2006), as well as trigger factor (Patzelt et al. 2001), small heat shock proteins (Lentze and Narberhaus 2004), and the bacterial translocation specific chaperone SecB (Knoblauch et al. 1999).

Binding of Hsp104 to hydrophobic peptides supports the hypothesis that, like other molecular chaperones, Hsp104 distinguishes misfolded proteins from their correctly folded conformers based on the exposure of hydrophobic amino acid side chains. We have previously reported that the composition of Hsp104-binding peptides is enriched in hydrophobic residues including Phe and Tyr, as well as in the charged amino acids Lys and Asp, and that the positions of Hsp104-interacting peptides from the globular domain of Sup35, when mapped onto a three-dimensional model of the domain, correspond to polypeptide segments that are only solvent-
exposed at their ends in the folded protein. In this study, we provide further evidence for this hypothesis by concluding that Phe, Tyr, Lys, and Asp contribute to the binding of a model peptide to Hsp104, and that substitution of these residues with a non-conservative amino acid abrogates binding to ATP-bound Hsp104. We have previously observed that p370 binds to Hsp104 at independent sites in both D1 and D2 (see 3.4.3) (Lum et al. 2008) and that binding was observed in both the ATP and ADP bound states. In future experiments, we will determine the nucleotide dependency of Hsp104 binding to p370 variant peptides using SPR.

A recent analysis of polyglutamine aggregation in yeast revealed that the inclusion of tyrosine residues within polyglutamine tracts enhances the fragmentation and mitotic inheritance of these self-assembling aggregates supporting the idea that Hsp104 recognizes peptide segments containing aromatic residues (Alexandrov et al. 2008). We speculate that this mode of recognition may also be important for the Hsp104-dependent maintenance of the yeast prions \([PSI^+], [PIN^+],\) and \([URE3]\). Experiments are currently underway to identify peptide segments in yeast prions that are crucial for interaction with Hsp104.

### 4.5.2 Prerequisites for substrate binding

In addition to investigating the elements within substrates that serve as recognition elements for Hsp104 binding, we also examined several structural features of Hsp104 that are crucial for rapid formation of the Hsp104-substrate complex. By SPR analysis, we found at least two prerequisites for rapid substrate acquisition \textit{in vitro} by Hsp104: an intact NTD, and ATP binding at D1.

Evidence supports the hypothesis that the NTD of Hsp104/ClpB modulates its interaction with protein aggregates (Liu et al. 2002; Tanaka et al. 2004; Barnett et al.). Our observation that rapid binding of substrates to Hsp104 requires an intact NTD is consistent with this hypothesis.
However, mutants of Hsp104 that lack an NTD are fully functional in *in vivo* and *in vitro* in refolding (Lum et al. 2008) and in providing thermostolerance in yeast (Hung and Masison 2006), processes that are dependent on the presence of a cognate Hsp70 chaperone system. This suggests that the NTD is, to some degree, dispensable for Hsp104 function. To explain this inconsistency, we propose that the role of the NTD becomes necessary only in the absence of Hsp70/40. This is supported by several lines of evidence. First, DnaK binds directly to ClpB (Schlee et al. 2004; Kedzierska et al. 2005) and can recruit ClpB to an aggregate surface (Acebron et al. 2009), suggesting that DnaK may function as an adaptor protein facilitating the formation of an initial ClpB-substrate complex (Haslberger et al. 2007). Secondly, elements in the NTD and the coiled-coil region of ClpB are crucial for mediating the DnaK-ClpB interaction (Kedzierska et al. 2005). Third, we observed that Hsp104 lacking an NTD binds weakly to a model unfolded protein whereas it is dispensable for the refolding of aggregated firefly luciferase in the presence of Hsp70/40. Fourth, mutations in conserved residues of the ClpB NTD reduce binding to casein, a model substrate that can be processed in an Hsp70-independent manner (Liu et al. 2002). Finally, efficient reactivation by ClpB and the DnaK cognate system of a strongly aggregated model substrate requires the ClpB NTD (Barnett et al. 2005). The exact mechanism by which the NTD supports binding of substrates remains unclear. Currently, no high resolution structural models of the Hsp104 NTD exist. However, a crystal structure of the orthologous ClpB NTD shows a hydrophobic groove on the surface of the domain that could potentially function to bind unfolded polypeptides (Li and Sha 2003). Furthermore, amino acid substitutions of residues that line this groove reduce ClpB binding to model substrate proteins (Tanaka et al. 2004; Barnett et al. 2005) and exhibit defects in aggregate refolding while retaining wild-type ATPase activity (Li and Sha 2003). Our provisional homology model of the Hsp104 NTD based on the ClpB structure contains an analogous hydrophobic groove (Fig 4.4).
Figure 4.4  The putative peptide binding groove located in the N-terminal domain of ClpB/Hsp104. Surface potential drawings of the (A) ClpB NTD structure (Li and Sha 2003) and (B) a homology model of the Hsp104 NTD structure based on the ClpB NTD using Swiss-Model (Guex and Peitsch 1997; Schwede et al. 2003). Negatively charged regions, red; positively charged regions, blue; hydrophobic regions, white. Circled in green is a putative peptide binding groove.
Determining the contributions of amino acid residues that line this groove in the binding of model substrates to Hsp104 will help to clarify the role of this domain and provide a framework from which additional biochemical studies can be designed. Further studies on the mechanism of substrate recognition by Hsp104 will advance our understanding of the role of its co-chaperones in suppressing and reversing protein aggregation.

In the absence of the Hsp70/40 co-chaperones, Hsp104 can still remodel protein aggregates. Under conditions that decelerate the ATPase activity of Hsp104, refolding of protein aggregates and prion fibrils has been observed (Doyle et al. 2007), indicating that Hsp104 can indeed bind directly to substrates without the assistance of adaptor proteins. Binding of a model unfolded protein is robust in the presence of an Hsp104\textsuperscript{trap} mutant that is locked in an ATP-bound conformation and is not detected in the presence of ADP-bound or nucleotide-free Hsp104, suggesting that Hsp104’s binding and release cycle is controlled by nucleotide hydrolysis and exchange (Bosl et al. 2005). Binding of an unfolded protein also stimulates the ATP hydrolytic activity of the normally quiescent NBD2 of Hsp104 (Schaupp et al. 2007). Under more dynamic conditions where ATP is freely hydrolyzing at either the D1 or D2 ATPase domains, we find that rapid binding of substrates occurs only when ATP is bound at D1 suggesting that this state represents the high affinity substrate binding state. Previously, we termed this state the “primed state” (Lum et al. 2008). A protein aggregate interaction at D1, whether spontaneously formed without the assistance of adapter proteins or delivered to Hsp104 by the Hsp70 system, is then likely sufficient to initiate unfolding and translocation.

Other structural elements in Hsp104 may be required for efficient substrate acquisition and transduction of an allosteric signal from D1 to D2. We have previously shown that stable binding of a model unfolded protein to ATP-bound Hsp104 requires flexible pore loops at D1
and D2 that are also crucial for thermotolerance and protein remodeling (Lum et al. 2004; Lum et al. 2008). However, the role of the axial channel loops in the binding of a model substrate under conditions where ATP is freely hydrolyzing has not yet been determined. Specific structural rearrangements that occur within Hsp104 that transmit the allosteric signal from D1 upon substrate binding to D2 where stimulation of ATP hydrolysis occurs are still unknown. Additional structural and biochemical data is required to explore this aspect of the protein unfolding mechanism of Hsp104.

4.6 Acknowledgements

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5 Future directions

In recent years, significant progress has been made in elucidating many aspects of the mechanism of protein disaggregation by Hsp104. When I first began my research on Hsp104, its cellular role was restricted to survival after heat stress and prion propagation. Recently, additional roles have been attributed to Hsp104. Yeast Hsp104 has been suggested to play a role in the asymmetric distribution of oxidatively damaged proteins and this function possibly involves remodeling the actin cytoskeleton (Aguilaniu et al. 2003; Tessarz et al. 2009), and the organization of yeast septin ring complexes (Schirmer et al. 2004). In plants, a chloroplast-targeted Hsp104 homolog was discovered to have housekeeping functions and is essential for chloroplast development (Myouga et al. 2006; Lee et al. 2007). As each of these functions likely involves the protein remodeling activity of Hsp104, a continued emphasis on understanding the biochemical and structural features of Hsp104 is key to future advancements in the Hsp100 field.

5.1 Short term goals – peptide binding to Hsp104

Currently, our method for the analysis of Hsp104 activity in vitro is the refolding assay. The substrate used, urea-denatured aggregated firefly luciferase, is non-uniform and amorphous and its refolding properties are sensitive to slight variations in the method of preparation. The utility of this assay is therefore restricted only as a qualitative indicator of Hsp104 function and cannot reliably assess other biochemical parameters of Hsp104 such as rates of substrate unfolding and chaperone to substrate stoichiometry. Studies of protein unfolding mechanisms in other Hsp100s have been greatly assisted by the ability to target natural and model monomeric substrates to the Hsp100. Both ClpA and ClpX recognize a short 9-amino acid C-terminal degradation signal peptide (SsrA tag) (Gottesman et al. 1998). Subsequent experiments with
GFP fused to SsrA have been very useful in the elucidation the mechanisms of these Hsp100s (Weber-Ban *et al.* 1999; Singh *et al.* 2000; Reid *et al.* 2001).

An analogous system can be developed to examine Hsp104s mechanism. In this thesis, I have established that the exposure of hydrophobic peptides upon protein misfolding is a key signal that initiates Hsp104-dependent protein remodeling. I therefore hypothesize that fusion of a high affinity Hsp104-binding peptide to GFP should target GFP to Hsp104 for unfolding and translocation. The unfolding process can be monitored in real-time by the loss of fluorescence intensity, as GFP fluorescence is strictly dependent on the proper folding of the molecule (Reid and Flynn 1997). Spontaneous refolding of unfolded GFP will be trapped by the addition of a GroEL “trap” mutant that irreversibly binds to non-native proteins and does not hydrolyze ATP (Weber-Ban *et al.* 1999). This assay would permit us to reliably determine, for example, how the amount of ATP consumed by Hsp104 is related to the rate of protein unfolding if the reaction is coupled to a spectrophotometric assay to measure ATP hydrolysis, as has been previously done with the ClpXP system (Burton *et al.* 2003). Furthermore, one could monitor the unfolding of substrates that vary in their intrinsic resistance to denaturation and simultaneously link this to ATP consumption. A GFP-titin-peptide substrate consisting of a GFP domain, the I27 domain from titin, and a high affinity Hsp104-binding peptide could be used. Amino acid substitutions in titin can be introduced to produce a spectrum of substrate proteins with decreasing stabilities from native to fully denatured forms (Kenniston *et al.* 2003).

Because misfolded, monomeric GFP refolds and becomes autofluorescent in the absence of chaperones, under conditions where GFP can be unfolded and induced to form aggregates, we can monitor the efficiency of GFP refolding when fused to an Hsp104 targeting sequence in reactions containing Hsp104 alone. The influence of a cognate Hsp70 system could be
determined by adding an Hsp70-targeting signal to GFP and measuring the kinetics of refolding in the presence of the complete bichaperone system and with Hsp104 alone as a control.

In addition, one could use dihydrofolate reductase (DHFR) as model substrate. DHFR bound to the folate analog methotrexate becomes stabilized and is prevented from translocating through the outer mitochondrial membrane (Gaume et al. 1998) as well as from degradation by the ClpAP protease (Lee et al. 2001). We predict that the stabilized DHFR/methotrexate complex, if targeted to Hsp104 by a peptide, will be trapped in an intermediate state that mimics the unfolding and translocation of a natural substrate. Sufficient quantities of this intermediate can be produced to analyze its biochemical properties (stability of the complex with and without nucleotide for example). Preliminary experiments fusing DHFR directly to a peptide tag as a C-terminal extension yielded fusion proteins that were insoluble. One can attempt to restore solubility by adding unstructured peptide-linkers of different lengths between DHFR and the Hsp104-binding peptide. If successful, this body of work will provide us with the tools to monitor in real time the unfolding process and to obtain direct evidence in support of an unfolding/threading model for Hsp104-mediated disaggregation. We can then contemplate characterizing the structural properties of the Hsp104-substrate complex using electron microscopy to visualize the position of DHFR when bound to Hsp104.

To engineer Hsp104 to specifically attack protein aggregates associated with disease, it would be useful to understand how Hsp104 recognizes its targets, whether through detection of polypeptide segments with particular amino acid composition, or through topological information displayed by protein aggregates that distinguish them from native complexes. The ability to target specific monomeric proteins with different inherent stabilities for unfolding or refolding would provide us with invaluable tools for studying the mechanism of Hsp104
function. Ultimately, it may be possible to predict which disease-associated aggregates might be amenable to Hsp104 disaggregation.

5.2 Long term goals – the structure of Hsp104

Clearly, the most contentious issue regarding Hsp104 function is the ambiguity regarding its quaternary structure. What will attract the attention of Hsp104 researchers in the immediate future is the generation of more structural information to help distinguish between the two dramatically different structural models derived from cryo-EM reconstructions from two independent groups (Wendler et al. 2007; Lee et al. 2010) (see 1.4.3.2).

To distinguish between the two radically different structural models of Hsp104, one may independently construct a structural model. Preliminary electron micrographs of an Hsp104 ‘trap’ mutant that is locked in an ATP-bound state (in collaboration with Dr. John Rubenstein, University of Toronto) provide promising images of the Hsp104 hexamer (data not shown) and indicate that a high resolution reconstruction of a model of the hexamer should indeed be possible.

Biochemical approaches to distinguish between the two different structural models of Hsp104 should also be employed. Currently in development is an Hsp104 mutant that lacks cysteine residues such that unique cysteines can be reintroduced in the molecule and will function as specific sites for chemical modification. Unique Cys residues will be introduced in positions along the coiled coil domain and the accessibility of these residues will be assessed by biotinylation of the free thiol. This way, we could differentiate one of the most glaring disparities between the two structural models of Hsp104 – whether the coiled-coil domain is situated on the exterior of the hexamer or intercalates between adjacent subunits.
A ‘cys-less’ Hsp104 mutant could also be useful in identifying structures that line the pathway for a translocating polypeptide in a series of fluorescence resonance energy transfer (FRET) experiments. Unique cysteines could be introduced along the axial channel and a fluorescent probe could be covalently attached to it. A fluorescently labeled model unfolded substrate such as RCMLa could then be used as a FRET pair. This type of experiment could tell us the distance a particular structure in the axial channel is from a substrate and therefore whether or not the structure is likely to participate in translocation. We will seek to corroborate this data with cryoEM studies of Hsp104 stably bound to RCMLa.

5.3 Final thoughts

Understanding the structural basis of linking ATP binding, hydrolysis and exchange in the AAA⁺ modules to conformational changes Hsp104 that drive disaggregation of disordered and ordered assemblies will contribute greatly to appreciating the mechanism, not just of Hsp100/Clp proteins, but perhaps many other AAA⁺ proteins as well. The inherent connectivity between the AAA⁺ modules both within and between subunits promises to make this a very challenging endeavor. The observed specificities in the bacterial and yeast bichaperone networks remain an enigma. Specifically, a potential role of DnaK/Hsp70 as a substrate adapter for ClpB/Hsp104 is especially attractive and it would be particularly satisfying to understand its determinants.
6 References


