Review

Hsp104 and ClpB: protein disaggregating machines

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Heat-shock protein 104 (Hsp104) and caseinolytic peptidase B (ClpB), members of the AAA+ superfamily, are molecular machines involved in disaggregating insoluble protein aggregates, a process not long ago thought to be impossible. During extreme stress they are essential for cell survival. In addition, Hsp104 regulates prion assembly and disassembly. For most of their protein remodeling activities Hsp104 and ClpB work in collaboration with the Hsp70 or DnaK chaperone systems. Together, the two chaperones catalyze protein disaggregation and reactivation by a mechanism probably involving the extraction of polypeptides from aggregates by forced unfolding and translocation through the Hsp104/ClpB central cavity. The polypeptides are then released back into the cellular milieu for spontaneous or chaperone-mediated refolding.

Introduction to Hsp104 and ClpB: AAA+ ATPases and members of the Clp/Hsp100 family of protein-remodeling machines

Many essential cellular functions require the action of molecular chaperones. These functions include the folding of newly synthesized proteins, refolding and reactivation of unfolded and misfolded proteins, assembly and disassembly of macromolecular protein structures, and targeting abnormal and inactive proteins for degradation [1,2]. Yeast heat-shock protein 104 (Hsp104) and its bacterial homolog caseinolytic peptidase B (ClpB) are molecular chaperones that have the ability to solubilize almost any protein that becomes aggregated after severe stress. Although not required under normal growth conditions, they can increase survival many thousand-fold when cells are exposed to extreme heat or other harsh stresses [3–6]. In addition, yeast Hsp104 is absolutely required for the inheritance of specific protein amyloids (see Glossary), called prions [7–9].

Hsp104 and ClpB are members of the AAA+ superfamily of ATPases (ATPases associated with various cellular activities) and homologs exist in fungi, bacteria, plants and eukaryotic mitochondria. The family is defined by the presence of a basic core of ~200–250 amino acids that comprises an α-helical domain and a Walker-type nucleotide-binding domain [10–12]. Hsp104 and its homologs belong to the Clp/Hsp100 family of AAA+ proteins. This family has the ability to remodel proteins in an ATP-dependent manner [13–15]. Clp/Hsp100 proteins are further divided into two classes (Figure 1a). Class 1 contains proteins with two AAA+ modules and includes Hsp104, ClpB and their close relatives mitochondrial Hsp78 and plant Hsp101. Also in class 1 are more distant relatives of Hsp104, including ClpA and ClpC. Class 2 is made up of proteins with one nucleotide-binding domain such as ClpX and heat-shock-like U (HslU). In addition, many Clp/Hsp100 chaperones, but not Hsp104 and ClpB, can associate with proteolytic components to form ATP-dependent proteases. For example, *Escherichia coli* ClpA, ClpX and HslU form ClpAP, ClpXP and HslUV, respectively [2,15].

For many of their protein disaggregating activities, Hsp104 and ClpB act in conjunction with the Hsp70/DnaK chaperone system [16–19]. Hsp70 in eukaryotes and DnaK in prokaryotes are members of another large, ubiquitous family of ATP-dependent molecular chaperones. The Hsp70/DnaK chaperone system reactivates inactive proteins and disassembles protein complexes in conjunction with two co-chaperones, Hsp40/DnaJ and nucleotide exchange factor (NEF)/GroP E (GrpE) [1,20]. This chaperone system functions in protein folding and remodeling under both non-stress and stress conditions.

Glossary

AAA+ superfamily of ATPases: the distinguishing feature of the AAA+ (ATPases associated with various cellular activities) family of proteins is a 200–250 amino acid ATP binding site containing several characteristic motifs, including Walker A, Walker B, sensor 1, sensor 2 and arginine finger motifs [10–12].

Amyloids: fibrous protein aggregates that share specific structural traits, including an exceptionally stable cross-beta quaternary structure with an extensive network of hydrogen bonds. Many human diseases are caused by the conversion of native proteins into an amyloid conformation, including Alzheimer's disease, Parkinson's disease, Huntington's disease, type II diabetes and prion diseases [77].

Clp/Hsp100 proteins: members of the AAA+ family of ATPases. They are hexameric protein machines that catalyze protein unfolding, disassembly and disaggregation in bacteria, plants and animals [2,5,15]. Some, but not all, associate with proteolytic components forming compartmentalized ATP-dependent proteases.

Hsp40/DnaJ: Hsp70/DnaK co-chaperones that facilitate substrate recognition by Hsp70/DnaK and stimulate Hsp70/DnaK ATP hydrolysis [1,20].

Hsp70/DnaK: family of heat-shock proteins present in almost all living organisms. Members of this family use the energy of ATP hydrolysis to bind and remodel client proteins. Hsp70/DnaK requires the help of co-chaperones Hsp40/DnaJ and NEF/GrpE to remodel substrate proteins efficiently [1,20].

NEF/GrpE: NEF (nucleotide exchange factor) of eukaryotes and GrpE of prokaryotes are Hsp70 and DnaK co-chaperones, respectively, that facilitate nucleotide exchange by Hsp70/DnaK [1,20].

Prions: conformationally modified proteins forming proteinaceous infectious particles. Prions cause a number of neurodegenerative diseases in mammals, including 'mad cow disease' in cows, Scrapie in sheep and Creutzfeldt-Jakob disease in humans [8].

Thermotolerance: originally defined as a short-lived state of enhanced heat resistance that is stimulated by exposure to mild heat stress [3]. It is also defined as thermoresistance to extreme heat stress in the absence of any pretreatment.

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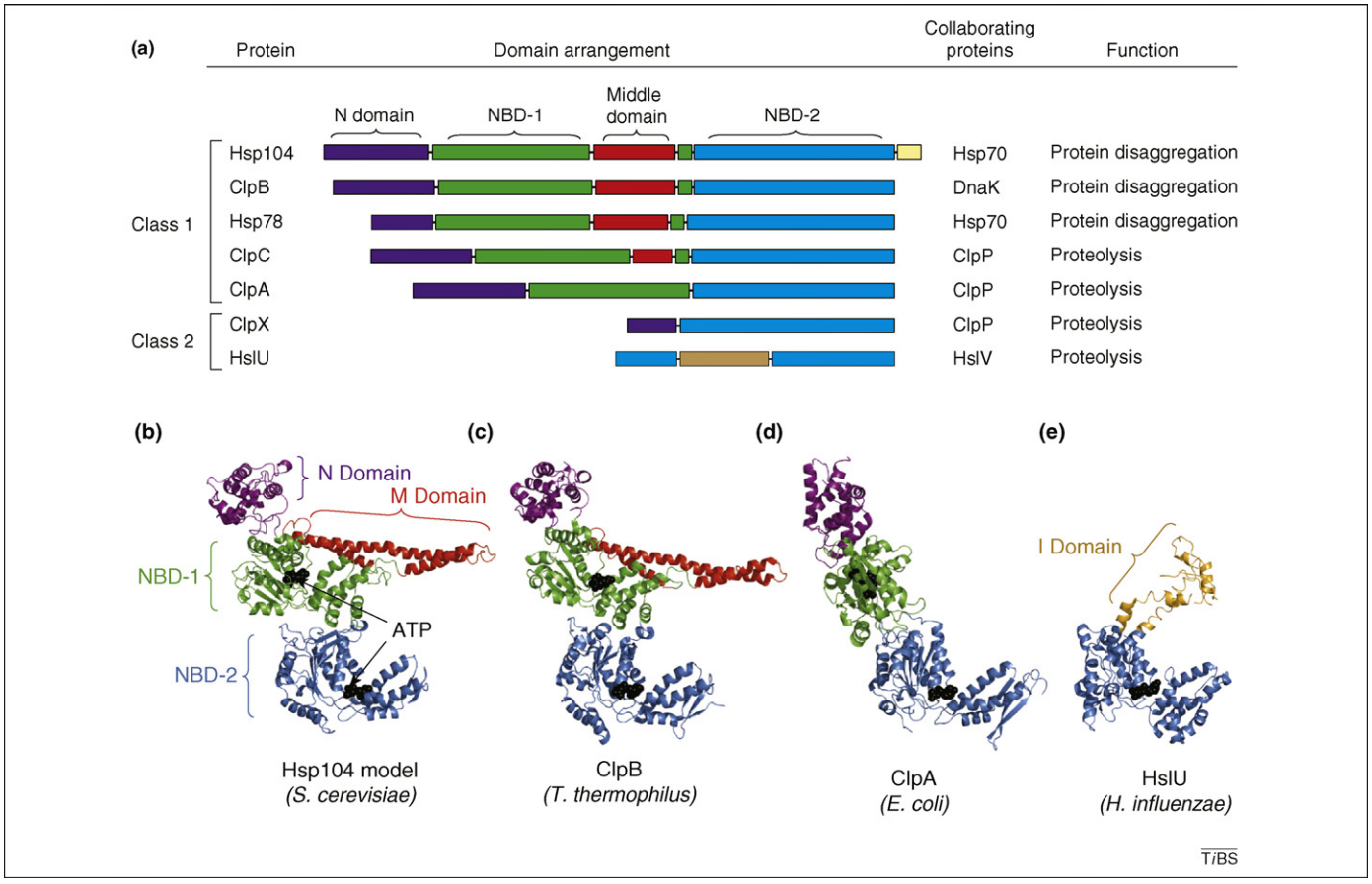

Figure 1. Domain organization of Clp/Hsp100 protomers. Both the domain organization and the structure of Clp/Hsp100 proteins are conserved in fungi, bacteria, plants and eukaryotes. **(a)** Class 1 Clp/Hsp100 proteins have two nucleotide-binding domains (NBDs), whereas class 2 proteins have a single NBD. The NBDs (NBD-1, green; NBD-2, light blue) are highly conserved, whereas the N domains (dark blue) have low sequence homology (the N-terminal domain of ClpX binds to Zn^{2+}, whereas HslU lacks an N domain but has an I domain [gold] that is inserted in its NBD). NBD-1 of Hsp104, ClpB, Hsp78 and ClpC is disrupted by a coiled-coil M domain (red) that varies in length and homology. Hsp104 has an additional unique C-terminal domain (yellow). The single NBD of ClpX and HslU is most homologous to NBD-2 of the class 1 ATPases. Hsp104, ClpB and Hsp78 are involved in protein disaggregation and act in conjunction with the Hsp70/DnaK chaperone system. ClpC, ClpA, ClpX and HslU interact with proteolytic components, ClpP or HslV, forming ClpCP, ClpAP, ClpXP and HslUV proteases. **(b)** Protomer model of *S. cerevisiae* Hsp104. The model was generated using Swiss-Model [81] and based on the crystal structure of *T. thermophilus* ClpB (PDB ID code 1qvr – chain C; www.rcsb.org) [23]. The C domain of Hsp104 is not shown on this model because ClpB lacks a similar domain. **(c)** Protomer structure of *T. thermophilus* ClpB (PDB ID code 1qvr – chain C) [23]. **(d)** Protomer structure of *E. coli* ClpA (PDB ID code 1ksf) [40]. **(e)** Protomer structure of *Haemophilus influenzae* HslU (PDB ID code 1e94) [41]. In (b-e), the N domain, NBD-1, NBD-2, coiled-coil M domain, C-terminal domain and I domain are shown, when present. ATP (black) is shown as a CPK model. All images were prepared using PYMOL (www.pymol.org).

The demonstration that Hsp104 mediates protein disaggregation has existed for <15 years [14]. Although much progress has been made in studying the proteins involved in disaggregation, the molecular mechanism of this process remains unclear. The aim of this review is to highlight the current understanding, both structural and mechanistic, of the Hsp104 and ClpB disaggregating machines.

Structure of Hsp104 and ClpB

Domain arrangement of Hsp104 and ClpB protomers

Hsp104 and ClpB, like other Clp/Hsp100 proteins and many AAA+ proteins, assemble into ring structures comprising six protomers [21,22]. The crystal structure of *Thermus thermophilus* ClpB has been solved and shows an N-terminal domain (N domain) followed by a nucleotide-binding domain (NBD-1), a middle domain (M domain) and a second nucleotide-binding domain (NBD-2) [23] (Figure 1a,c). Because of its high homology to ClpB, a similar model of Hsp104 can be generated (Figure 1a,b). Whereas all class 1 Clp/Hsp100 proteins share highly homologous NBDs, the N and M domains, when present, are only distantly related. For example, ClpC has a shortened M domain and ClpA lacks an M domain altogether (Figure 1a,d). By comparison, the single NBD of class 2 Clp/Hsp100 proteins is most homologous to NBD-2 of class 1 proteins (Figure 1a,e).

The NBDs of Hsp104 and ClpB are AAA+ modules containing the characteristic Walker A and B motifs and sensor-1 and -2 motifs (Figure 2; Table 1). Mutants harboring substitutions in the conserved AAA+ motifs exhibit defects in thermotolerance, prion propagation and chaperone activity [24–28]. Both NBDs bind and hydrolyze ATP. Binding of ATP by the NBDs stabilizes both the oligomeric state [21,22] and interactions with substrate [29–31]. ATP hydrolysis provides the energy for protein remodeling, although the mechanism is not fully understood, and is discussed in more detail later.

The unique M domain distinguishes Hsp104 and ClpB from other Clp/Hsp100 proteins. It is inserted in NBD-1 and consists of two antiparallel coiled-coils that resemble a two-bladed propeller [23] (Figure 1b,c). The M domain has an important yet undefined role because a deletion of or

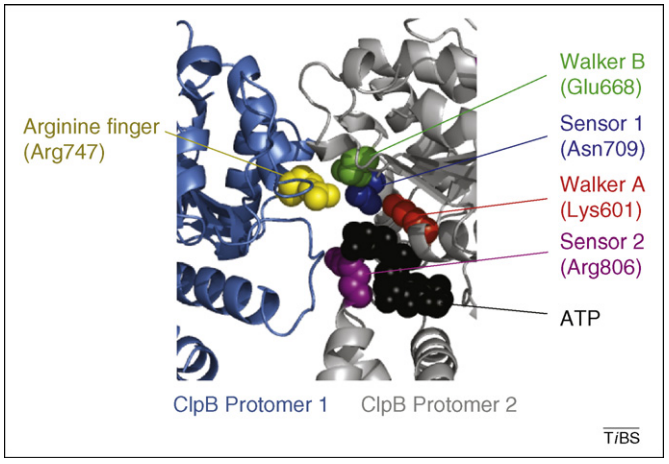

Figure 2. Model of ClpB showing the subunit interface. A close-up view of two adjacent subunits of *T. thermophilus* ClpB (PDB ID code 1qvr – chain C) is shown [23,46]. Neighboring subunits are shown in ribbon diagrams, one in gray and one in blue. Amino acid residues from conserved motifs involved in nucleotide binding are shown as CPK models: arginine finger (yellow), Walker A (red), Walker B (green), sensor 1 (blue) and sensor 2 (purple). ATP is shown in black as a CPK model. Image was prepared using PYMOL (www.pymol.org).

mutations in this domain can result in a loss of protein remodeling activity for Hsp104 and ClpB [25,32], in addition to defects in thermotolerance and cell division [33].

The Hsp104 N domain is not essential for yeast thermotolerance or prion propagation, but it is required to cure prions by Hsp104 overexpression [34]. Similarly, the ClpB N domain is not absolutely required for bacterial thermotolerance [25]. Moreover, for the majority of substrates studied, ClpB N-terminal deletion mutants exhibit the same *in vitro* protein remodeling activities as their wild-type counterparts [25,35]. There are indications that the ClpB N domain is likely to be important for interaction with specific substrates and might have a crucial role when the DnaK system is limiting [36].

Hsp104 contains a small 38 amino acid C-terminal domain (C domain) downstream of NBD-2 that is not present in ClpB. This region has been implicated in thermotolerance and hexamer assembly [37,38].

Hexameric structures of Hsp104 and ClpB

Although the functional structure of Hsp104 and ClpB is a hexamer, the crystal structure of *T. thermophilus* ClpB solved by Tsai, Lee and colleagues [23] shows three monomers arranged in a spiral. Therefore, other techniques have been used to arrive at molecular models of Hsp104 and ClpB hexamers.

Electron microscopy and single-particle reconstructions of yeast Hsp104 (with a sensor-1 mutation [Asn728Ala] in NBD-2) have been carried out recently by Saibil, Lindquist and colleagues [39]. The particle, which was formed in the presence of ATPγS, is a hexamer with a three-tiered structure; one tier is formed by the N domain, one by NBD-1 and the third contains NBD-2 and the C domain [39] (Figure 3a,d). The cryo-electron microscopy (cryo-EM) maps of Hsp104 also show a large central cavity with an apical but not a distal pore [39].

A symmetric hexameric molecular model of Hsp104 has been built using the cryo-EM maps and a monomer model [39] based on known X-ray structures from bacterial homologs, ClpB [23] and ClpA [40]. This hexameric model of Hsp104 is remarkably different from other AAA+ proteins, the oligomeric structures of which were determined directly by X-ray crystallography, including HslU [41,42] and p97 (also called valosin-containing protein) [43]. One striking difference is that Hsp104 has a large central cavity of ~78 Å in diameter [39], whereas ClpB and other Clp proteins possess narrower axial cavities or channels of ~25 Å [41–45]. Another conspicuous difference is that, in Hsp104, the conserved arginine fingers in both NBD-1 and NBD-2 are positioned on the exterior of the molecule [39]. By contrast, the arginine fingers of other AAA+ proteins contact the ATP binding site of the adjacent subunit [10–12] (Figure 2). It was proposed that conserved arginines in the M domains, which in the Hsp104 model are positioned in the interior of the hexameric protein, could be involved in nucleotide contact substituting for the typical arginine fingers in other AAA+ proteins. In a variety of thermotolerance assays, mutations in the M-domain arginines caused defects similar to those observed for mutations of the conserved arginine fingers in NBD-1 and NBD-2 [39].

Table 1. Conserved motifs in Clp proteins.

Motif	Conserved residues	Common substitution	Effect of mutation	Refs
Walker A	Gly-Xaa-Xaa-Xaa-Xaa-Gly-Lys[Thr/Ser][a] P-loop directly interacts with phosphates of ATP	Lys→Ala	Inhibits nucleotide binding; can affect oligomeric state	[5,21,24,25,78]
Walker B	hhhhAsp-Glu[b] Forms contacts with ATP, Mg^{2+} and water to coordinate ATP hydrolysis	Glu→Gln/Ala	Inhibits ATP hydrolysis; does not inhibit ATP binding	[5,25,78]
Sensor 1	Polar residue Interacts with the γ-phosphate of ATP	Asn/Thr→Ala	Inhibits ATP hydrolysis	[5,26,78]
Sensor 2	Gly-Ala-Arg 'Sensor and substrate discrimination' sequence that interacts with the γ-phosphate of ATP	Arg→Ala/Met	Inhibits ATP hydrolysis; can affect ATP binding; can affect oligomeric state	[5,24,25,78,79]
Arginine finger	Arg Forms part of the NBD for the adjacent subunit	Arg→Ala	Inhibits ATP hydrolysis for most AAA+ proteins; can affect oligomeric state	[12,25]
Pore loop	Tyr Loop facing the central channel in the hexamer; involved in substrate binding and translocation	Tyr→Xaa	Inhibits substrate binding and translocation; has little effect on ATP hydrolysis or oligomerization	[50,72,80]

[a]Xaa stands for any amino acid.
[b]h stands for any hydrophobic amino acid.

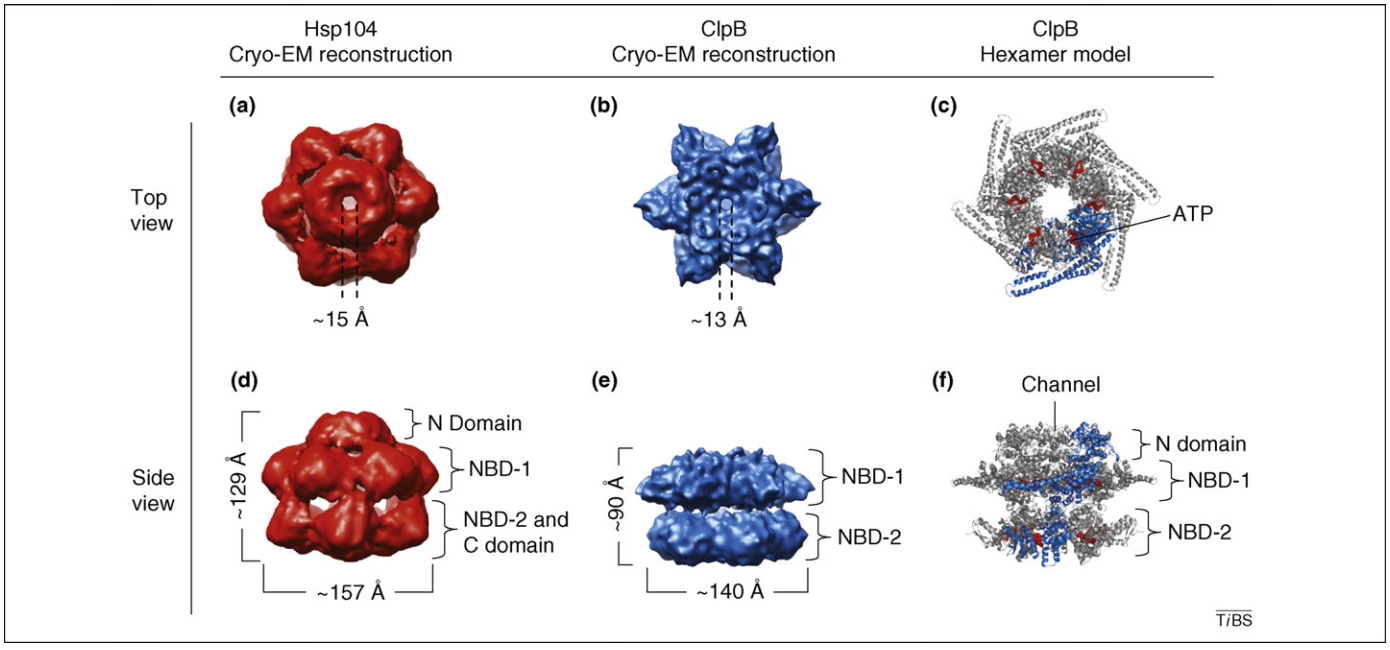

Figure 3. Models of Hsp104 and ClpB. Hexameric models of Hsp104 and ClpB are compared. **(a,d)** Three-dimensional cryo-EM reconstruction (13 Å) of *S. cerevisiae* Hsp104 (Asn728Ala, a mutant defective in ATP hydrolysis) prepared without fixatives in the ATPγS-bound form (EMD-1358) [39], shown as top (a) and side (d) views and prepared using Chimera (www.cgl.ucsf.edu/chimera). **(b,e)** Three-dimensional cryo-EM reconstruction (11.2 Å) of full-length *T. thermophilus* ClpB (Glu271Ala/Glu668Ala, a double mutant that is able to bind but not hydrolyze ATP) prepared in the presence of glutaraldehyde with ATP bound (EMD-1244) [44] shown as top (b) and side (e) views [44] and prepared using Chimera. The N domains and portions of the M domains were not observed. **(c,f)** *T. thermophilus* ClpB (wild-type) hexamer model with bound ATP [46]. The image was prepared using PYMOL (www.pymol.org). A single protomer is shown in blue and the position of ATP in red as a CPK model.

The M-domain mutants had altered ATPase activity, with a decrease in activity for two mutants similar to the mutated arginine fingers, but increased activity for one [39]. These experiments support the model but are also consistent with alternative models.

Reconstructions of electron microscopic images of a *T. thermophilus* ClpB mutant with Walker B mutations in NBD-1 and NBD-2 (Glu271Ala and Glu668Ala, respectively) with ATP bound show a two-tiered hexameric structure made up of NBD-1 and NBD-2 with an apical, but not a distal, pore [23,44] (Figure 3b,e). Because the N-terminal domains were not detected and the coiled-coil M domains were only partially visible, it was suggested that these domains have a great deal of flexibility.

An atomic model has been built of ClpB using the hexameric cryo-EM maps and the crystal structure of a *T. thermophilus* ClpB protomer [23,44,46] (Figure 3c,f). The AAA+ modules are positioned as in other AAA+ proteins [10,11] and, as mentioned earlier, vastly different from in the Hsp104 model [39]. The model of ClpB has the coiled-coil M domains located on the outer surface of the oligomeric ring of NBD-1, in stark contrast to the interior position of the M domains in the Hsp104 model [39,44]. Mutagenesis and crosslinking experiments support this arrangement of the M domains on the exterior of the hexamer but do not eliminate other possible arrangements [23,47]. In addition, the ClpB model has a much smaller central cavity than Hsp104 [23,39,44]. The startling differences in structural models between these two very similar proteins remain unexplained.

Collaboration of Hsp104 and ClpB with the Hsp70/DnaK chaperone system

In vivo, Hsp104 and ClpB function with the Hsp70 and DnaK chaperone systems, respectively, to dissolve protein aggregates [14,48]. *In vitro*, the two chaperone systems act together to disaggregate insoluble aggregates that neither chaperone is able to efficiently act on alone [16–19]. Moreover, ClpB and the DnaK system act synergistically to remodel substrates that each can act on separately [49].

Interaction of Hsp104/ClpB with Hsp70/DnaK

The role of the Hsp70/DnaK system in protein remodeling and disaggregation reactions with Hsp104 and ClpB is not fully understood, but recent findings are providing clues. The Hsp70/DnaK system is essential early in the disaggregation process [47,50,51], possibly assisting in the extraction of polypeptides from aggregates by presenting unstructured regions of the aggregate to Hsp104/ClpB. Additionally, Hsp70/DnaK might modulate the ATPase cycle of Hsp104/ClpB to facilitate protein remodeling by Hsp104/ClpB [49]. The Hsp70/DnaK system can also act late in the disaggregation process by assisting in reactivation of protein substrates that fail to spontaneously refold after the action of Hsp104/ClpB. In summary, the precise division of labor between Hsp70/DnaK and Hsp104/ClpB has not been established.

In vitro observations showing species specificity for the Hsp104/ClpB component and the Hsp70/DnaK component indicate that there is direct physical contact between the two chaperones. For example, using purified proteins, yeast Hsp104 requires yeast Hsp70 and *T. thermophilus* ClpB requires *T. thermophilus* DnaK to reactivate substrates; neither yeast Hsp104 nor *T. thermophilus* ClpB is able to act with *E. coli* DnaK [16,52]. Similarly, *E. coli* ClpB acts with *E. coli* DnaK but not with yeast cytosolic Hsp70 [53]. However, notable exceptions are that *E. coli* ClpB functions with yeast mitochondrial Hsp70 [53] and yeast Hsp104 functions with human Hsp70 [54,55].

Although a direct demonstration of physical complexes between Hsp104 and Hsp70 has not been observed, ClpB from *T. thermophilus* associates with immobilized *T. thermophilus* DnaK in a species-dependent manner [52]. Moreover, this interaction does not involve the N domain, but it does require the hexameric form of ClpB [52]. The M domain of *E. coli* ClpB is important for the collaboration between ClpB and DnaK because amino acid substitutions in helix-3 of the M domain inhibit the collaboration [47].

Protein remodeling by Hsp104 and ClpB independently of the Hsp70/DnaK system

Because Hsp104/ClpB functions together with the Hsp70/DnaK chaperone system in most *in vitro* disaggregation reactions, it has been difficult to tease out the specific role for each of the two ATP-dependent molecular chaperones. However, current research has demonstrated that Hsp104 and ClpB can remodel proteins independent of the Hsp70/DnaK system and has provided a better understanding of the enzymatic capabilities of these important protein remodeling machines.

The initial evidence that Hsp104 alone remodels substrate proteins came from the work of Shorter and Lindquist [56]. They found that *in vitro* high concentrations of Hsp104 block the assembly of yeast prion proteins into large insoluble fibers by eliminating the oligomeric intermediates that nucleate fiber assembly. The reaction was observed with Sup35, a yeast translation terminator protein, and Ure2, a regulator of nitrogen metabolism in yeast [56,57]. Moreover, at high concentrations Hsp104 disassembles preformed insoluble Sup35 and Ure2 fibers [56,57]. In both reactions, Hsp104 uses the energy of ATP hydrolysis to remodel prions. Conversely, at low concentrations of Hsp104, the rate of fiber formation is stimulated [56,58]. This reaction requires ATP binding, but not hydrolysis because AMPPNP, a nonhydrolyzable ATP analog, supports the reaction [56]. These studies indicate that Hsp104 has the innate ability to remodel proteins and disassemble specific ordered aggregates. However, the reaction involves more factors *in vivo*, including Hsp70 and its co-chaperones, small heat-shock proteins and Hsp90 co-chaperones [9,59]. Very recently the interplay between Hsp104 and the Hsp70 chaperone system has been investigated *in vitro* [60].

Hsp104 and ClpB alone also remodel substrates other than prions *in vitro.* They catalyze the disaggregation of heat-denatured soluble aggregates as shown recently by Doyle *et al.* [61]. Additionally, they remodel RepA, a protein that initiates DNA replication of P1 plasmids in *E. coli,* by converting inactive dimers into active monomers [61]. The same substrates are also remodeled by ClpA and the DnaK system alone [13,61,62]. These protein-remodeling activities of Hsp104/ClpB are evoked when mixtures of ATP and ATPγS, a slowly hydrolyzed and non-physiological ATP analog, are used [61]. Protein remodeling is also exhibited by Hsp104/ClpB mutant proteins defective in nucleotide hydrolysis at either NBD-1 or NBD-2 [61]. These experiments indicate that slowing ATP hydrolysis at some of the nucleotide-binding sites elicits the innate protein-remodeling activity of the chaperones. However, Hsp104/ClpB is not able to disaggregate insoluble aggregates under these *in vitro* conditions, and *in vivo* the NBD mutants do not enable the cells to develop thermotolerance [25–27]. Nonetheless, these tools have enabled the study of the innate remodeling activities of Hsp104 and ClpB without the complication of additional chaperones and co-chaperones.

Mechanism of action of Hsp104/ClpB in collaboration with Hsp70/DnaK

Recent evidence indicates that Hsp104 and ClpB function by a mechanism that involves unfolding and translocating of substrate polypeptides through the central channel of the hexamer as demonstrated for ClpA and ClpX [2,15,63–67]. Bukau and colleagues [50,68] engineered the ClpP interaction site of ClpA into Hsp104 and ClpB, enabling them to interact with ClpP and function as two-component ATP-dependent proteases, named Hsp104–ClpA–P-loop (HAP) and ClpB–ClpA–P-loop (BAP), respectively. HAP and BAP, when associated with ClpP (referred to as HAP-P and BAP-P), unfold and translocate polypeptides through their central channels and into the cavity of the associated ClpP protease, as with ClpAP and ClpXP, where degradation occurs [50,68], [2,15]. Furthermore, BAP-P has the ability to unfold and degrade an unstructured domain when that region is between two stably folded domains that are not degraded [69]. Consequently, BAP is able to process protein loops [69] as does ClpX [70]. It has been suggested that substrates with unfolded domains flanked by stably folded domains might be released from ClpB by hexamer dissociation, a process that occurs in under a minute [69,71]. However, the *in vivo* relevance of disassembly has not been determined. Importantly, *in vivo* HAP and BAP restore thermotolerance when expressed in *hsp104* or *clpB* deletion strains, whereas co-expression of HAP or BAP with ClpP inhibits the development of thermotolerance and cell recovery after heat stress. Thus, disaggregation and reactivation of proteins is essential for cell survival after extreme heat stress, whereas degradation of aggregated proteins is insufficient [50,68].

An unfolding and translocating mechanism of protein remodeling by Hsp104 and ClpB was further substantiated by a study showing that both proteins have the intrinsic ability to unfold natively folded proteins independent of the Hsp70/DnaK system [61]. In addition, the products of disaggregation by Hsp104/ClpB in the absence of Hsp70/DnaK are unfolded polypeptides [54,61]. In these experiments the innate unfolding activities were elicited using mixtures of ATP and ATPγS or by the use of mutants with defective ATP hydrolytic activity. It is likely that these *in vitro* conditions enable the substrate to be held (a process requiring ATP binding but not hydrolysis) and, at the same time, unfolded (a process requiring ATP hydrolysis). *In vivo* synchronizing substrate holding and unfolding might be a function of the Hsp70/DnaK chaperone system.

Another line of evidence supporting the translocation mechanism comes from mutational analyses of the conserved tyrosine-containing loops that are present in the AAA domains of all Clp/Hsp100 proteins [10,11]. In ClpB, the pore loops of both NBD-1 and NBD-2 are modeled to project into the axial channel of the hexamer [23,44]. ClpB and Hsp104 containing mutations in the pore-loop tyrosine

of either NBD-1 or NBD-2 have decreased translocation and disaggregation activity *in vitro*, owing to defective interactions with the substrate [29,50,68,72,73]. Genetic evidence indicating the importance of the central channel of Hsp104 in prion propagation and thermotolerance is consistent with the biochemical results [34,74]. It is difficult to reconcile the biochemical and genetic studies of Hsp104 demonstrating polypeptide translocation and the importance of the pore loops in light of the structural data showing that Hsp104 possesses a large central cavity [39].

How aggregates are recognized and presented to Hsp104/ClpB for unfolding remains a puzzling question. As mentioned earlier, the Hsp70/DnaK system acts in the early stages of protein disaggregation, possibly facilitating substrate recognition by Hsp104/ClpB. However, *in vitro* in the absence of Hsp70/DnaK, Hsp104/ClpB is able to stably bind substrates in a reaction that can be detected when ATPγS is used in place of ATP or when mutants defective in ATP hydrolysis are used [29,31,50]. Thus, Hsp104/ClpB has the innate ability to interact with substrates independently of Hsp70/DnaK. By analogy to ClpA and ClpX [2,15], substrate recognition by Hsp104 and ClpB is likely to involve binding to an unstructured region. Owing to the complexity of aggregates, recognition motifs would be expected to occur commonly. Either an unstructured end of the substrate [54,61] or an unstructured loop, as discussed earlier [69], can enter the Hsp104/ClpB channel.

Determining how ATP hydrolysis is coupled to protein unfolding and translocation associated with disaggregation is central for understanding the mechanism of action of Hsp104/ClpB. However, characterizing the ATP hydrolytic cycle of Hsp104/ClpB has been difficult because there are 12 nucleotide-binding sites per hexamer. Moreover, by analogy to ClpX [75], it is likely that hundreds of cycles of ATP hydrolysis are required to extract a single polypeptide from an aggregate. For ClpXP, Sauer, Baker, and colleagues [76] have shown that the power stroke for unfolding and translocation is generated by ATP hydrolysis in a single subunit of the hexameric ring, supporting a mechanism involving a probabilistic sequence of ATP hydrolysis. It remains to be determined whether or not the probabilistic mechanism extends to Hsp104 and ClpB. Importantly, if Hsp104 and ClpB act by a similar mechanism, the hexameric models described earlier that have been constructed based on sixfold symmetry will not reflect the actual structures of the active asymmetric proteins.

Working models for the mechanism of action of Hsp104/ClpB with the Hsp70/DnaK system

Our current model for disaggregation by the collaborative action of Hsp104/ClpB with Hsp70/DnaK assumes that each chaperone functions by the same mechanism it uses when acting alone, but the two activities are now productively coupled. It is anticipated that Hsp104 and ClpB, two highly homologous proteins, act by similar molecular mechanisms; however, this remains to be demonstrated.

Taken together, recent work discussed earlier indicates that the mechanism of action of Hsp104 and ClpB is similar to that of other Clp/Hsp100 ATPases (Figure 4a). Exposed unstructured regions, not necessarily at the ends of the protein [69], are recognized in a reaction requiring ATP binding but not hydrolysis [Figure 4a(i)]. The Hsp70/DnaK system and Hsp104/ClpB are usually both required at this step for protein disaggregation. In the absence of the Hsp70/DnaK system, the innate remodeling activities of Hsp104/ClpB can be detected by using mutants defective in ATP hydrolysis at one of the NBDs or by using a mixture of ATP and ATPγS [54,61]. Polypeptides are unfolded by forcible translocation through the central channel of Hsp104/ClpB using the energy of ATP hydrolysis [Figure 4a(ii)]. ATP hydrolysis is thought to induce conformational changes in Hsp104 and ClpB resulting in movement of the pore loops that directly interact with substrates and reside in the pore [44,73]. The unfolded polypeptides are released in a reaction also requiring ATP hydrolysis [31] [Figure 4a(iii)]. However, the entire polypeptide need not be unfolded; partially unfolded polypeptides can be released, perhaps by a mechanism involving disassembly of Hsp104/ClpB [69,71]. Dependent upon the specific substrate protein, some unfolded polypeptides are able to refold spontaneously, whereas others require the assistance of molecular chaperones [Figure 4a(iv)].

When Clp/Hsp100 proteins are associated with a protease component they bind to substrates. The substrates are unfolded by the force associated with translocation of the polypeptide through the central channel into the proteolytic chamber where degradation occurs [2,15] (Figure 4b). The process of degradation elucidated for HAP-P and BAP-P [50,68] is almost identical to that of the other Clp proteases. However, when the substrate is an aggregate or a folded protein, HAP-P and BAP-P require the Hsp70/DnaK system for degradation [50,68].

In vitro Hsp104 might remodel prion proteins by a mechanism similar to that discussed for remodeling by Clp/Hsp100 proteins. Prion proteins are unique Hsp104 substrates and might be recognized through a specific recognition signal in an unstructured region [Figure 4c(i)]. Interestingly, *E. coli* ClpB is unable to act on yeast prion proteins, emphasizing a difference in substrate specificity of the two remodeling proteins [56]. Based on the known unfolding and translocating capabilities of Hsp104, prion proteins are likely to be unfolded and translocated through Hsp104 [Figure 4c(ii–iv)]. Removal of a protomer from the amyloid fiber severs the fiber, thereby increasing the number of assembly sites (seeds) for fiber formation. *In vivo*, the Hsp70 system, co-chaperones and small heat-shock proteins are likely to be involved in modulating these reactions [9,59].

A combined mechanism involving Hsp104/ClpB and the Hsp70/DnaK system is more speculative. One model to be considered is that Hsp104/ClpB is the primary protein disaggregating machine, forcibly extracting and unfolding polypeptides from aggregates via translocation through its axial channel [Figure 4d(i–iii)]. The unfolded polypeptides are released to spontaneously refold or to be acted upon by other chaperones [Figure 4d(iv)]. Hsp70/DnaK probably assists in presenting substrates to Hsp104/ClpB [47,50,51] and might coordinate substrate binding and translocation by regulating ATP hydrolysis by Hsp104/ClpB.

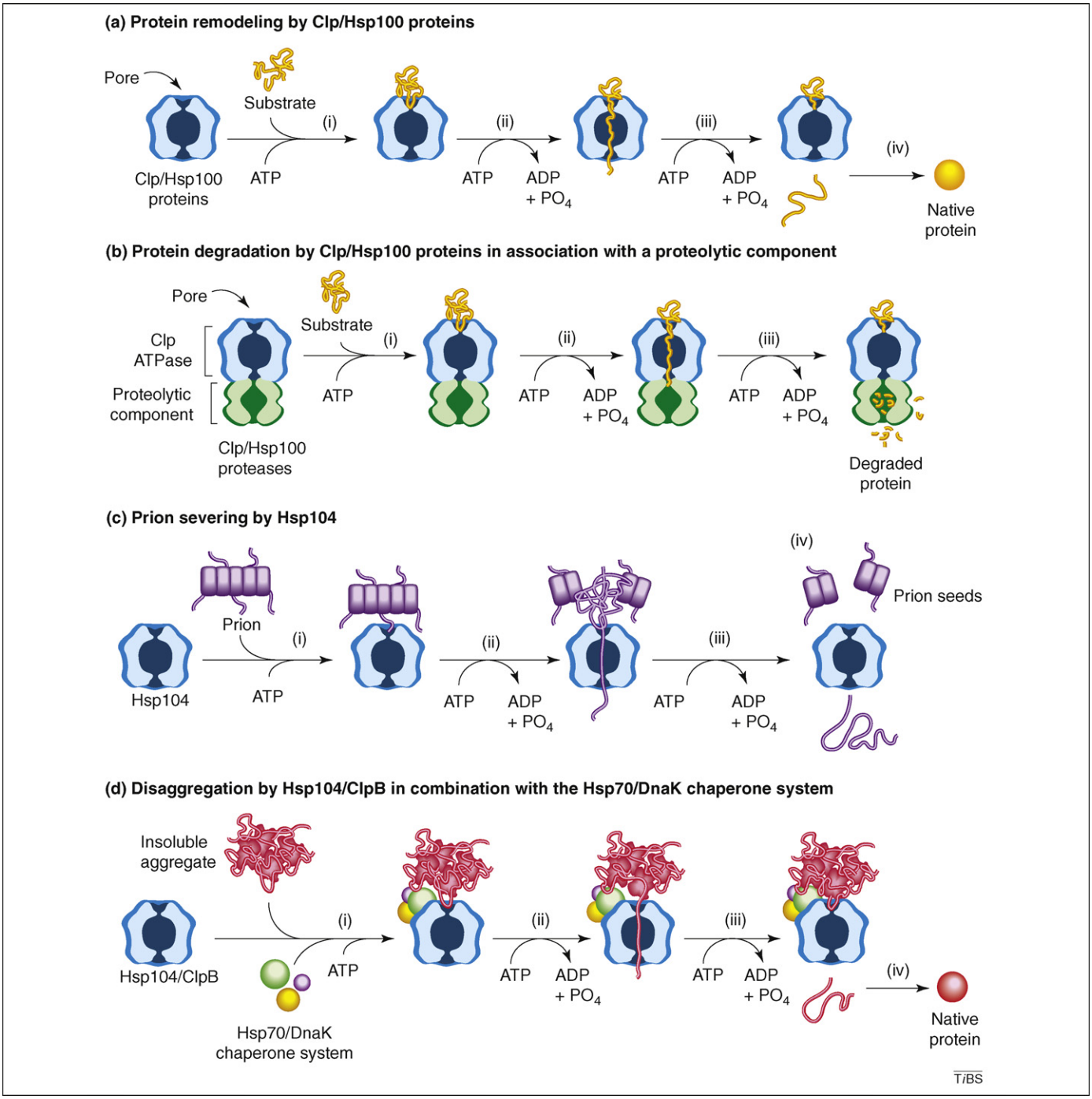

Figure 4. Models for the mechanisms of action of Clp/Hsp100 proteins. Unified models are depicted, indicating that the Clp/Hsp100 family of molecular chaperones functions similarly when remodeling, degrading or disaggregating proteins. **(a)** Protein remodeling by Clp/Hsp100 proteins. Substrates, such as specific proteins, multimeric proteins and small aggregates, interact via unstructured regions containing recognition motifs with Clp/Hsp100 proteins, including ClpA, ClpX, HslU, ClpC, Hsp104 and ClpB (i). Nucleotide binding stabilizes the hexameric structure of the Clp/Hsp100 proteins and interactions between substrates and the Clp/Hsp100 protein. Substrates are unfolded by forcible translocation through the central channel of Clp/Hsp100 proteins using the energy of ATP hydrolysis (ii). The unfolded polypeptides are released (iii), a step induced by ATP hydrolysis, and refold spontaneously (iv) or with the help of molecular chaperones, depending on the specific substrate. As discussed in the text, the intrinsic unfolding and translocating activity of Hsp104/ClpB is elicited by using mixtures of ATP and ATPγS, *in vitro* conditions that enable substrate binding (a reaction requiring ATP binding without hydrolysis and promoted by ATPγS) and protein unfolding (a reaction requiring ATP hydrolysis and promoted by ATP). **(b)** Protein degradation by Clp/Hsp100 proteins in association with a proteolytic component. When Clp/Hsp100 proteins, including ClpA, ClpX, HslU, ClpC, BAP and HAP are associated with a partner peptidase forming ClpAP, ClpXP, HslUV, ClpCP, BAP-P and HAP-P, the Clp/Hsp100 component recognizes, unfolds and translocates substrate polypeptides (i,ii) processively into the chamber of the proteolytic component where degradation occurs (iii). BAP and HAP are mutant ClpB and Hsp104 proteins, respectively, that were engineered to contain the ClpP interaction site of ClpA and can function with ClpP as two-component ATP-dependent proteases [50,68]. Although HAP-P and BAP-P are able to translocate and degrade peptides and unfolded proteins alone, the Hsp70/DnaK chaperone system is required for degradation of native proteins and aggregates. **(c)** Prion severing by Hsp104. *In vitro*, Hsp104 is capable of severing yeast prions [56,57]. In the presence of ATP, Hsp104 is likely to interact with prion fibers via an exposed unstructured tag on a protomer (i). By analogy with the mechanism for Hsp104 and HAP when acting on other substrates (a,b), prion protomers are extracted from the fiber by unfolding and translocating through the channel of Hsp104 and released using the energy of ATP hydrolysis (ii,iii). The process results in twice the number of available ends, or seeds, for further growth of fibers (iv). Although the Hsp70 system is not required for this reaction *in vitro*, it and other co-chaperones and small heat-shock proteins modulate prion propagation *in vivo* [9,59]. **(d)** Disaggregation by Hsp104 and ClpB in combination with the Hsp70/DnaK system. The Hsp70/DnaK chaperone system interacts with Hsp104/ClpB and acts before or in conjunction with Hsp104/ClpB [47,50,51]. Hsp70/DnaK is likely to assist in presenting substrates to Hsp104/ClpB (i) and might additionally coordinate ATP hydrolysis by Hsp104/ClpB. Hsp104 and ClpB perform the primary work of disaggregation. They remove polypeptides from the aggregate by forcible translocation of the polypeptides through their central channels (ii) and release the unfolded polypeptides (iii) to refold spontaneously or with assistance of other chaperones, which might include Hsp70/DnaK (iv).

Concluding remarks

Hsp104 and its homologs are protein disaggregating machines working side by side with other chaperones and proteases to ensure protein quality control in the cell. In most situations, Hsp104 and ClpB work in collaboration with the Hsp70/DnaK chaperone system to remodel and disaggregate substrates ranging from specific ordered amyloid fibers (prions) to highly disordered insoluble protein aggregates. Although much has been learned about this important class of protein disaggregating machines, there are still many unanswered questions. One challenge of future work will be to determine whether the mechanism of protein disaggregation by Hsp104 is analogous to the mechanism for ClpB and other homologs. Furthermore, how substrates are recognized by Hsp104/ClpB and how ATP hydrolysis is coupled to disaggregation by Hsp104/ClpB need to be addressed. Importantly, the interplay between Hsp104/ClpB and the Hsp70/DnaK system remains to be explained.

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