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Aggregate reactivation mediated by the Hsp100 chaperones

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Abstract

Hsp100 family of molecular chaperones shows a unique capability to resolubilize and reactivate aggregated proteins. The Hsp100-mediated protein disaggregation is linked to the activity of other chaperones from the Hsp70 and Hsp40 families. The best-studied members of the Hsp100 family are the bacterial ClpB and Hsp104 from yeast. Hsp100 chaperones are members of a large super-family of energy-driven conformational “machines” known as AAA+ ATPases. This review describes the current mechanistic model of the chaperone-induced protein disaggregation and explains how the structural architecture of Hsp100 supports disaggregation and how the co-chaperones may participate in the Hsp100-mediated reactions.

Keywords

protein aggregation; aggregate reactivation; molecular chaperone; ClpB; Hsp104; AAA+ ATPase

The biological function of ClpB/Hsp104

Protein aggregation can be defined as a non-physiological association of misfolded or partially folded polypeptides [1–4]. Unlike protein folding/unfolding, which can be considered reversible, aggregation is practically irreversible under native-like conditions, which implies that, once initiated, it produces polypeptide conglomerates of increasing size that are soluble at first, but eventually precipitate. Morphologically, the aggregates represent either amorphous particles or ordered amyloid fibers, both types enriched in β -structure [1, 4]. *In vivo*, misfolded and mildly aggregated proteins are usually degraded by cellular protease systems [5, 6], but excessive aggregation can stall the protein degradation machinery with severe consequences including cell death [7, 8]. Many cellular pathologies and human diseases are associated with accumulation of aggregates in cells and tissues: from sickle-cell anemia, Alzheimer’s, Huntington, and Parkinson’s disease, to prion diseases [2]. Protein aggregation is also a common nuisance in biochemical laboratories and an obstacle in efficient production of recombinant proteins in biotech industry.

To deal with protein misfolding and aggregation, cells produce a number of proteins known as molecular chaperones. Since misfolding and aggregation are likely to occur during

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cellular stress, it is not surprising that all heat-shock protein families (Hsp) display some form of chaperone activity [9, 10]. Individual chaperone families apply different mechanisms to affect conformation of protein substrates. Well-studied “conventional” chaperones (Hsp60/GroEL, Hsp70/DnaK, small Hsps) recognize and interact with partially folded proteins, and consequently inhibit their aggregation. Selected aggregates can be rescued by the DnaK/DnaJ/GrpE system [11]. However, conventional chaperones do not provide a robust disaggregation activity.

Lindquist and coworkers were the first to show that yeast cells possess a capability to dissociate protein aggregates after a non-lethal heat stress [12]. The yeast “disaggregase” activity is associated with the protein Hsp104 [13]. It has been found next that ClpB is a functional ortholog of Hsp104 in bacteria and that ClpB reactivates aggregated proteins in cooperation with the DnaK/DnaJ/GrpE system [14–16]. Many different aggregated bacterial proteins can be rescued by the concerted action of ClpB and DnaK/DnaJ/GrpE [17]. The remarkable disaggregation activity makes Hsp100 proteins (ClpB/Hsp104) unique and distinct from the conventional molecular chaperones.

Hsp100 chaperones are found in bacteria, yeast, and plants, but not in animals or humans. Hsp100 are in general dispensable under normal growth conditions, but become essential for survival during severe heat shock [18–20]. Thus, one can speculate that the *hsp100* gene evolved in the organisms, which must periodically survive a severe environmental stress. In addition to the aggregate-reactivation activity, Hsp104 has been implicated in the propagation of yeast prions [21]. Interestingly, the prion remodeling capability is not conserved among Hsp100 proteins because the bacterial chaperone ClpB cannot replace the yeast Hsp104 in prion propagation *in vivo* [22].

Recently, evidence has been accumulating for the essential function of Hsp100 in pathogenic microorganisms. ClpB is required for invasiveness and/or in-host survival of a number of bacterial and protozoan pathogens including *Porphyromonas gingivalis* [23], *Mycoplasma pneumoniae* [24], *Francisella tularensis* [25], *Enterococcus faecalis* [26], *Mycobacterium tuberculosis* [27], *Plasmodium falciparum* [28], and *Leishmania donovani* [29]. Those results suggest that pathogens are exposed to a significant stress during infection of a host and that stress-induced protein aggregation may be detrimental to their survival. No biochemical studies on the activity of ClpB in pathogens have been reported yet.

Structural architecture of ClpB/Hsp104

Hsp100 proteins (ClpB/Hsp104) belong to the AAA+ superfamily of “ATPases associated with various cellular activities” [30, 31]. AAA+ ATPases are found in both prokaryotic and eukaryotic cells and are involved in a variety of functions, such as protein quality control (refolding, disaggregation, and degradation), membrane fusion and vesicular transport, DNA replication and repair, and cytoskeletal regulation. AAA+ proteins can be described as “molecular machines” that use energy from the hydrolysis of ATP to induce changes in macromolecular structure or to disassemble macromolecular complexes. The function and dysfunction of many AAA+ ATPases are linked to human diseases [31].

A characteristic feature of AAA+ ATPases is ~250 amino-acid long ATP-binding sequence module. The AAA+ module contains a number of conserved motifs including Walker A and B, sensor-1 and -2, and the arginine-finger motif and folds into a two-domain structure with the ATP-binding site located between the subdomains [31]. Many AAA+ ATPases, including Hsp100 proteins contain in their sequence two AAA+ modules (called D1 and D2). Interestingly, each AAA+ module of ClpB/Hsp104 apparently evolved independently from the other one [32] which suggests a convergent evolution of the tandem structure.

In addition to the ATP-binding modules, AAA+ proteins contain less-conserved domains that specify the cellular localization and/or substrate preference. ClpB/Hsp104 contains the N-terminal domain of ~150 a.a., which contributes to the aggregate-binding affinity [33]. Disaggregation of some substrates does not require the N-terminal domain of ClpB [33, 34]. *In vivo*, the N-terminal domain becomes essential for the optimal growth of bacteria at high temperature in the absence of the functional DnaK [35]. Characteristically, ClpB/Hsp104 also contains a coiled-coil domain inserted in D1, which is not found in other AAA+ ATPases [36, 37]. The exact function of this inserted region (often called the middle domain of ClpB) is unknown besides several hints described in the later sections of this review.

A common structural property of AAA+ ATPases is the formation of oligomeric rings, which are the biologically active structural units [38]. Nucleotide binding stabilizes the ring structure, which is consistent with the location of the ATP binding sites at the subunit interfaces. Most ring-type AAA+ structures show a six-fold symmetry with seven- or eight-member rings also documented [39]. Some AAA+ rings are stable under physiological conditions, while others dynamically assemble and disassemble [40]. In the absence of nucleotides, ClpB associates into a heptameric ring, which converts into a hexamer upon binding of ATP or ADP [41]. The mechanistic role of the apparent heptamer-hexamer “switch” in ClpB is currently unknown. The highly dynamic nature of the oligomers and the apparent instability of the ring are most likely the reasons for the non-physiological trimer found in the crystal structure of ClpB from *Thermus thermophilus* [37].

The crystal structure of ClpB shows the head-to-tail arrangement of D1 and D2 in each ClpB monomer (see Fig. 1) [37]. The N-terminal domain is connected to D1 with an unstructured linker. The middle domain contains two internal coiled-coils and forms an elongated structure reminiscent of a “dual-blade propeller” [37]. In agreement with other published AAA+ structures, each AAA+ module of ClpB consists of a larger α/β and a smaller α -helical domain.

Since the ring-like oligomers of Hsp100 were not observed in a crystal form, cryoEM image reconstruction proved critical for creating structural models of the biologically active oligomers. The reconstructed hexamer structure of *T. thermophilus* ClpB shows a double-layer cylinder with the top and bottom layers built of six D1 and D2 modules, respectively [37]. The N-terminal domains, which are connected to D1, were not apparently resolved in the cryoEM image averaging, which suggests a high mobility of those domains in the hexameric ClpB. Fig. 1 shows the homology model of *E. coli* ClpB based on the *T. thermophilus* hexamer structure. In the model, the orientation of the N-terminal domain corresponds to one of the conformations found in the ClpB trimer crystal structure [37]. A similar arrangement of domains to that found in bacterial ClpB has been recently described for the hexameric Hsp104 from *Saccharomyces cerevisiae* [42].

The top and bottom views of the structural model reveal a narrow channel at the center of the ClpB hexamer (see Fig. 1). The channel is surrounded by structural elements of the large subdomains of D1 and D2. The characteristic coiled-coil middle domain is located at the outer surface of the cylinder. The coiled-coil undergoes an apparent motion in response to nucleotide binding [43]. The small D2 subdomain (see the bottom view in Fig. 1) forms extensive contacts with the neighboring subunit within the ring, which are essential for the hexamer stability [44]. A ring of the N-terminal domains crowns the ClpB cylinder and surrounds the entrance to the central channel (see the top view in Fig. 1).

The structure of hexameric ClpB shown in Fig. 1 is consistent with those of other AAA+ proteins, which were crystallized in their full oligomeric form [45, 46]. Importantly, the model is in agreement with the biochemical data which place the nucleotide binding sites

between the subunits in such way that the Walker A and B motif from one of the protomers and the Arg-finger from the neighboring protomer interact with the nucleotide. Moreover, the model places the conserved “channel loop” motives of ClpB/Hsp104 [47] inside the central channel where they can interact with substrates (see below).

The Lindquist and Saibil groups reported an alternative cryoEM-based structural model of yeast Hsp104 [48]. In the alternative model of the hexameric ring, the D1 module of each protomer appears rotated by $\sim 45^\circ$ and D2 by $\sim 90^\circ$ relative to the conventional AAA+ orientation (see Fig. 1). As the consequence, the middle domain of Hsp104 docks between the subunits of the ring, which expands the cylinder size and creates an internal cavity that replaces the narrow channel shown in Fig. 1. The N-terminal domain of Hsp104 in this model interacts with the middle domain, which may restrict its mobility. The possibility of a significant structural difference between the bacterial ClpB and the yeast Hsp104 appears attractive because it might explain the inability of ClpB to process yeast prions. However, it is not clear why two different cryoEM analyses of Hsp104 gave conflicting structural models [42, 48]. It should be noted that the domain rotation proposed in the expanded-cavity model places the conserved AAA+ motifs in non-canonical positions, which suggests an alternative non-conserved mechanism of substrate processing.

The mechanism of protein disaggregation

The major progress in elucidating the mechanism of disaggregation mediated by Hsp100 chaperones has been made when Bukau and coworkers discovered that ClpB translocates substrate polypeptides through its central channel during aggregate reactivation (see Fig. 2) [49]. By modifying the “bottom” surface of the ClpB hexamer, Bukau and coworkers engineered a ClpB complex with the peptidase ClpP, in which the ClpB channel exit was connected to the entrance to the ClpP proteolytic chamber. Such modification of ClpB induced degradation of its substrates, which unequivocally demonstrated the threading of polypeptides through the ClpB channel during their disaggregation.

Since the small diameter of the channel does not allow threading of structured polypeptides, the ClpB substrate becomes gradually unfolded as it passes through the channel [50]. The substrate translocation and its forced unfolding require energy, which is supplied from the hydrolysis of ATP and then transduced through still uncharacterized conformational changes in D1 and D2 into a progressive motion of the substrate. The motion of the substrate is most likely mediated by movements of the unstructured loops in D1 and D2 that are exposed inside the channel [51]. The intrinsic instability of the ClpB ring allows the chaperone to dissociate from a polypeptide that contains stable domains and resists unfolding (see Fig. 2) [52]. Unlike a related bacterial AAA+ ATPase ClpA, which delivers the unfolded substrate to the associated peptidase ClpP for degradation [53], ClpB releases the unfolded substrate into solution. Later, an analogous mechanism of substrate translocation during the aggregate and prion processing has been found for Hsp104, which is consistent with the structural equivalency of the bacterial and fungal Hsp100 [54].

The elegant mechanism of ClpB/Hsp104 explains how inactive and apparently insoluble protein aggregates are gradually resolubilized. The soluble unfolded substrates extracted from aggregates can subsequently refold into native structures either spontaneously or with the assistance of other chaperones (see below), but they may also misfold and re-aggregate. The above mechanism explains why an over-production of ClpB in *E. coli* does not suppress, but rather increases protein aggregation [55]. Thus, cells must maintain a balance between the unfoldase activity of Hsp100 and the protein folding capacity of other chaperones in order to achieve the physiological protein homeostasis.

Substrate processing in the central channel of ClpB/Hsp104 is consistent with the emerging consensus of the mechanism of ring-forming AAA+ ATPases, which involves either complete or partial translocation of the substrates through the AAA+ channel [56]. Indeed, unstructured loops exposed inside the channel that may mediate substrate threading are among the AAA+ signature motifs [31, 47]. Various AAA+ ATPases may differ in the mechanism of energy transduction between multiple ATP-binding sites and the substrate-binding motifs. For ClpB/Hsp104, a number of studies suggest the existence of an allosteric network of linkages between the 12 nucleotide-binding sites within the hexameric ring and the substrate-processing loops [57–64]. Importantly, it has been established that Hsp100 interact with their substrates with high affinity in the ATP-bound state. However, the mechanism of energy transduction in Hsp100 proteins from the ATP binding/hydrolysis to substrate extraction is still unknown.

ClpB/Hsp104 must distinguish between aggregates and properly folded proteins. The substrate specificity of Hsp100 is important because a non-specific unfoldase activity might not be beneficial *in vivo*. Since protein aggregates, unlike degradation targets, are not tagged by any known mechanism, Hsp100 should recognize aggregate-specific surface motifs that do not occur on the surface of folded proteins. The physical/chemical nature of such aggregate-specific motifs is currently unknown. Peptide-library studies revealed that ClpB preferentially binds to positively charged and aromatic amino acids [65]. Whether such residues are enriched at the surface of growing aggregates where they could mediate the Hsp100 binding remains to be investigated. It has been established so far which parts of the ClpB structure are involved in substrate binding: the conserved residues near the entrance to the ClpB channel in D1 [65] and in the N-terminal domain of ClpB [33, 66]. The location of those motifs is in agreement with the D1-to-D2 direction of substrate translocation. No interaction has been found so far between aggregated substrates and the outer surface of the Hsp100 cylinder, including the coiled-coil domain. The coiled-coil domain may, however, play a regulatory role in aggregate reactivation by Hsp100/70/40 (see the text below and the recent review [67]).

Role of the co-chaperones

Since Hsp100 generate unfolded polypeptides from aggregated particles, but do not apparently assist in their refolding, it is not surprising that a complete reactivation of aggregated substrates requires help from the “conventional” chaperones [13, 15]. However, in the engineered ClpB/ClpP complex, the substrate release is blocked, but the disaggregation remains DnaK/DnaJ-dependent [49]. This important result implies that DnaK/DnaJ play an essential role before substrate threading through the ClpB channel. Indeed, the ClpB association with aggregates under conditions of the ATP turnover depends on DnaK/DnaJ [68]. The “upstream” activity of Hsp70/40 occurs in addition to any refolding of substrates that can be assisted by those co-chaperones after the substrates are released from Hsp100. Curiously, the cooperation between Hsp100 and Hsp70/40 is apparently “species specific”, i.e. bacterial DnaK/DnaJ do not cooperate with yeast Hsp104 and *vice versa* [13, 22, 69]. It should be stressed, however, that Hsp100 bind to aggregated substrates even in the absence of Hsp70/40 [33, 70].

The obligatory role of the Hsp70/40 co-chaperones in the Hsp100-mediated disaggregation was questioned by the discovery that some aggregates can be rescued by Hsp100 alone [42, 71]. The Hsp70/40-independent reactivation of aggregated GFP was observed under conditions of partial inactivation of the ClpB ATPase, which was achieved either by supplying ATP with the non-hydrolyzable ATP γ S or by using ClpB variants with mutations in the AAA+ motifs [71]. It could be hypothesized that partial deceleration of the ClpB ATPase produces a better coordinated or less wasteful threading engine that extracts GFP

from aggregated particles. The Hsp70/40-independent aggregate reactivation was also achieved by insertion of an extraneous domain (T4 lysozyme) into the Hsp104 middle domain [42, 72]. The apparent “gain” in the Hsp104 activity is most likely unrelated to the lysozyme activity, but it underscores a possible role of the middle domain in regulation of Hsp100. Indeed, some mutations in the middle domain of ClpB stimulate the ATPase, but inhibit aggregate reactivation [73].

The above results suggest that the role of Hsp70/40 co-chaperones in the Hsp100-mediated aggregate reactivation may include regulation of the Hsp100 ATPase. Partial “poisoning” of ClpB could mimic such regulation in the absence of the co-chaperones [71, 72]. Importantly, a direct interaction between Hsp100 and Hsp70/40 might account for the apparent “species specificity” in those multi-chaperone systems. Interactions between ClpB and DnaK were detected *in vitro* [74, 75] and *in vivo* [76]. The interaction of the two chaperones involves the substrate binding site of DnaK [75] and the middle coiled-coil domain of ClpB [76]. By studying the ClpB/Hsp104 chimeras, it has been found that the coiled-coil domain is responsible for the “species specificity” of the cooperation with Hsp70/40 [72, 76]. It still needs to be tested whether the Hsp70/40 binding to the middle domain of Hsp100 modulates the latter’s activity.

The hypothesis of a direct stimulation of Hsp100 by Hsp70/40 during aggregate reactivation implies that a complex of the chaperones might interact with aggregated substrates. Since Hsp70 is active as a monomer and has a single substrate-binding site, it should not bind to aggregates while it interacts with the middle domain of Hsp100. However, an observation of the substrate-bound multi-chaperone complex proved difficult. When ClpB and DnaK were chromatographed with large aggregates, only one of the chaperones predominantly bound to the substrate: ClpB displaced DnaK in the presence of ATP and DnaK displaced ClpB in the presence of ADP (S. Kedzierska-Mieszkowska and M. Zolkiewski, unpublished results). This result is consistent with the nucleotide preference of either chaperone for substrate binding and suggests that DnaK may compete with ClpB for sites at the surface of an aggregate instead of regulating ClpB and stimulating its aggregate-reactivating function.

Liberek and coworkers proposed an alternative hypothesis for the role of the co-chaperones in aggregate reactivation [77, 78]. Since pre-incubation of the aggregates with DnaK/DnaJ accelerates their subsequent reactivation by ClpB, it has been suggested that DnaK/DnaJ performs the initial disaggregation steps before ClpB can take over the substrates. Such prerequisite activity of DnaK/DnaJ suggests that the concentration of ClpB bound to aggregates should increase in the presence of DnaK/DnaJ. However, the opposite was observed: DnaK competed with ClpB for binding to the aggregates and the amount of bound ClpB decreased in the presence of DnaK/DnaJ [79]. This result indicates that DnaK/DnaJ do not apparently expose new ClpB binding sites at the aggregate surface, but they might increase the productivity of the ClpB interaction that leads to substrate extraction (see the next section below). Thus, the proposed “substrate-prepping” role of DnaK/DnaJ needs further exploration.

Postulate of a rate-limiting step in aggregate reactivation

E. coli produces two isoforms of ClpB: the full-length ClpB95 and the truncated ClpB80, which does not contain the N-terminal domain [19]. The role of the truncated ClpB isoform in aggregate reactivation has been discovered only recently [79]. It was found that ClpB95 and ClpB80 synergistically cooperate and together show several-fold higher aggregate-reactivation rates *in vivo* and *in vitro* than either ClpB95 or ClpB80 alone. ClpB95 and ClpB80 associate into hetero-hexamers, which form with a higher efficiency than homo-hexamers of ClpB95 [79]. Curiously, interactions between ClpB95 and ClpB80 neither

produce a more efficient binding of the chaperone to aggregated substrates nor cause faster ATP hydrolysis during substrate threading. Thus, it was proposed that a reaction step that occurs after the binding of ClpB to an aggregate and before substrate threading through the channel is rate limiting in aggregate reactivation [79]. The ClpB95/80 hetero-hexamers might accelerate such intermediate step.

Fig. 2 shows the current model of the Hsp100-mediated protein disaggregation. Based on the recent results, we postulate that the ATP-induced binding of a substrate to ClpB is not a direct prerequisite for substrate threading. We propose that ClpB must commit to translocating the substrate and that the rate of the commitment step can be controlled by multiple factors, as explained below.

The postulate of the ClpB commitment step is consistent with detailed studies of the substrate engagement by the channel structures [73]. Whereas aggregates efficiently bind to ClpB in the absence of DnaK/DnaJ, they do not apparently interact with the mobile channel loop in D1 unless DnaK/DnaJ are included. This result suggests that the commitment of ClpB to extract a polypeptide from an aggregate might correspond to the engagement of the substrate by the D1 channel loop (see Fig. 2). Moreover, the D1 loop interactions with aggregated substrates were inhibited by an amino-acid substitution in the coiled-coil middle domain [73]. This result demonstrates, again, a regulatory role of the ClpB middle domain in aggregate reactivation.

Computational simulations of the ClpB oligomers suggested that the mobility of the substrate-binding N-terminal domain is suppressed in the ClpB95 homo-hexamer and becomes enhanced in the ClpB95/ClpB80 hetero-hexamers [79]. Thus, the N-terminal domain mobility might account for the increased rates of aggregate reactivation by ClpB95/80. Altogether, the published results suggest that the rate of extraction of polypeptides from aggregated particles may be controlled by several factors including: *a.* transient interactions of DnaK/DnaJ with the aggregate and/or with the middle coiled-coil domain of ClpB; *b.* conformational changes and/or motions of the middle domain; and *c.* the mobility of the N-terminal domain. How the above factors control and facilitate the access of an aggregated polypeptide to the ClpB channel will be a subject of future studies.

Final remarks

Hsp100 family (ClpB, Hsp104) adds a robust protein disaggregation activity to the inventory of mechanisms used by the cellular protein quality control. Although animal cells do not contain Hsp100, it can be expected that understanding the Hsp100 mechanism might help in designing ways of controlling or reversing protein aggregation linked to human diseases [80]. Indeed, the expression of Hsp104 in animal models of Parkinson and Huntington disease showed beneficial effects of the yeast chaperone on animal survival and neural degeneration [81–83]. On the other hand, a physiologically-sufficient control of protein aggregation in animal cells may occur without the canonical Hsp100 and may be mediated by the network of other chaperones, such as an extensive family of Hsp70 [84]. Furthermore, understanding the cooperation between Hsp100, Hsp70/40, and possibly other molecular chaperones will help in controlling aggregation during production of recombinant proteins. As described in this article, an outline of the Hsp100-mediated disaggregation is now available (see Fig. 2), but uncovering the mechanistic details will require further efforts.

Highlights

Hsp100 chaperones support heat-shock survival of bacteria, yeast, and plants.

Hsp100 reactivate aggregated proteins in cooperation with Hsp70/40.
 Hsp100 chaperones (ClpB/Hsp104) belong to the AAA+ family of ATPases.
 Hsp100 use energy from ATP to extract single polypeptides from aggregated particles.

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Fig. 1.

The structural model of hexameric ClpB from *E. coli* (side, top, and bottom views). The individual domains in each ClpB monomer are indicated: N-terminal domain (red), D1 AAA + module (green), middle coiled-coil domain (magenta), D2 AAA+ module large sub-domain (blue), D2 AAA+ module small C-terminal sub-domain (orange). The homology model of the *E. coli* ClpB monomer was built from the structures of the *E. coli* ClpB N-terminal domain [85], *E. coli* D1 AAA+ module [86], and chain B from the trimeric structure of *T. thermophilus* ClpB [37] using MODELLER software [87, 88]. Conformation of the channel loops in D1 and D2 (visible in the top and bottom views), which were not resolved in the template structures, was refined with the automated loop-modeling module

[89]. The hexamer structure was obtained by assembling six monomers into a ring and performing a 500-step energy minimization with CHARMM and Generalized Born solvent model [90, 91].

Fig. 2.

The proposed mechanism of protein disaggregation mediated by Hsp100. Left: nucleotide-dependent association of monomeric Hsp100 into a cylinder-shaped hexamer. Top: ATP-stimulated binding of an aggregate (red) to the hexameric Hsp100 produces an uncommitted disaggregase complex. The formation of the uncommitted complex does not require the co-chaperones (see text). The postulated rate-limiting step (red arrow) leads to a committed disaggregase state, in which an aggregated polypeptide (blue) becomes engaged by the channel loops. The efficiency of the commitment step may be controlled by the mobility of the N-terminal domain, the conformational changes in the middle domain, and the interactions of the co-chaperones with the aggregate and/or the middle domain of Hsp100 (see text). Bottom: Non-productive dissociation of the Hsp100 hexamer from an aggregate which resists disaggregation and a productive release of an unfolded polypeptide after translocation through the Hsp100 channel.