

Mechanistic and Structural Insights into the Prion-Disaggregase Activity of Hsp104

Elizabeth A. Sweeny and James Shorter

Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Correspondence to James Shorter: jshorter@mail.med.upenn.edu

<http://dx.doi.org/10.1016/j.jmb.2015.11.016>

Edited by A. Martin

Abstract

Hsp104 is a dynamic ring translocase and hexameric AAA+ protein found in yeast, which couples ATP hydrolysis to disassembly and reactivation of proteins trapped in soluble preamyloid oligomers, disordered protein aggregates, and stable amyloid or prion conformers. Here, we highlight advances in our structural understanding of Hsp104 and how Hsp104 deconstructs Sup35 prions. Although the atomic structure of Hsp104 hexamers remains uncertain, volumetric reconstruction of Hsp104 hexamers in ATP γ S, ADP-AIF $_x$ (ATP hydrolysis transition-state mimic), and ADP via small-angle x-ray scattering has revealed a peristaltic pumping motion upon ATP hydrolysis. This pumping motion likely drives directional substrate translocation across the central Hsp104 channel. Hsp104 initially engages Sup35 prions immediately C-terminal to their cross- β structure. Directional pulling by Hsp104 then resolves N-terminal cross- β structure in a stepwise manner. First, Hsp104 fragments the prion. Second, Hsp104 unfolds cross- β structure. Third, Hsp104 releases soluble Sup35. Deletion of the Hsp104 N-terminal domain yields a hypomorphic disaggregase, Hsp104 $^{\Delta N}$, with an altered pumping mechanism. Hsp104 $^{\Delta N}$ fragments Sup35 prions without unfolding cross- β structure or releasing soluble Sup35. Moreover, Hsp104 $^{\Delta N}$ activity cannot be enhanced by mutations in the middle domain that potentiate disaggregase activity. Thus, the N-terminal domain is critical for the full repertoire of Hsp104 activities.

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Introduction

Protein disaggregases are potential therapeutic agents to reverse protein misfolding, aggregation, and amyloidogenesis that underpin several fatal human neurodegenerative disorders [1–15]. Moreover, amyloid fibrils highly abundant in human seminal fluid enhance HIV (human immunodeficiency virus) infection [16–18] and agents that reverse their assembly could help prevent HIV transmission [19–22]. The ability to reverse protein aggregation and recover pure, functional protein could also have important applications in basic research as well as in the purification and storage of valuable pharmaceutical proteins [23,24]. However, despite these potential basic, therapeutic, and pharmaceutical applications, we are still only beginning to understand the structural and mechanistic basis of protein disaggregases.

In yeast, a ring-shaped translocase and hexameric AAA+ protein, Hsp104, couples ATP hydrolysis to the disassembly and reactivation of disordered aggregates, preamyloid oligomers, amyloids, and prions [1,6,25–30]. Typically (but not always), Hsp104 disaggregase activity is optimal in the presence of the Hsp70 chaperone system [1,26,27,31,32]. Protein disaggregation is driven by Hsp104 coupling ATP hydrolysis to the partial or complete translocation of substrate across its central channel to solution by conserved tyrosine-bearing pore loops [33–37]. Hsp104 activity can be potentiated by mutations at specific positions in the coiled-coil middle domain (MD) or in nucleotide-binding domain 1 (NBD1) of Hsp104 (Fig. 1a) [7,38–41]. Potentiating mutations enable Hsp104 to dissolve fibrils formed by human neurodegenerative disease proteins such as TDP-43, FUS, and α -synuclein and mitigate neurodegeneration

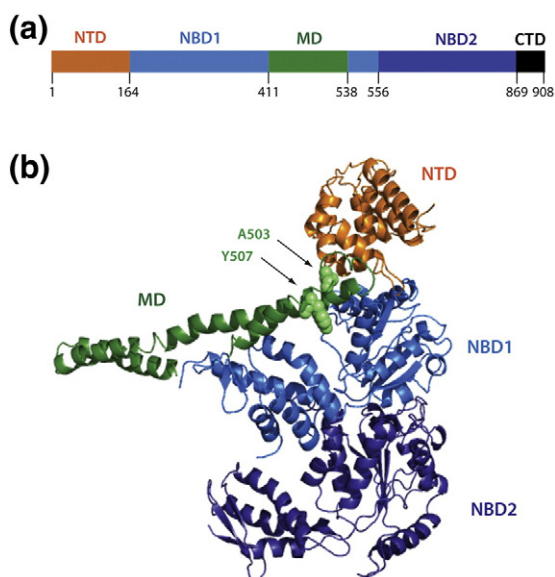


Fig. 1. Domain architecture and predicted monomeric structure of Hsp104. (a) Domain organization of the Hsp104 monomer (upper panel). NTD shown in orange, NBD1 shown in light blue, MD shown in green, NBD2 shown in dark blue, and CTD shown in black. (b) The separate domains of Hsp104 color-coded as in (a) were homology modeled and fitted together. NTD is from PDB ID: 1khy and NBD1, MD, and NBD2 are from PDB ID: 1qvr. Note the positions of A503 and Y507 in helix 3 of the MD.

under conditions where wild-type Hsp104 is inactive [7]. Intriguingly, Hsp104 is absent from metazoa but is found in all other eukaryotes and in all eubacteria and some archaeobacteria [42–45]. It has only recently been appreciated that metazoa rely upon the Hsp110, Hsp70, and Hsp40 chaperone system to disaggregate and reactivate proteins [5,14,46–51]. However, Hsp104 further stimulates the activity of this system [5,14,49]. Thus, Hsp104 could represent a disruptive technology to enhance metazoan proteostasis to counter fatal neurodegenerative disease or HIV infection [1–7,19,20].

Hsp104 confers several selective advantages in yeast. First, regulated amyloid remodeling by Hsp104 enables yeast to exploit prions for diverse adaptive purposes [52–57]. Second, Hsp104 confers tolerance to diverse thermal and chemical stresses by recovering natively folded protein from disordered aggregates harboring denatured proteins and by disassembling aggregated structures harboring functional proteins [25,26,58–60]. Third, Hsp104 promotes yeast longevity by regulating asymmetric partitioning of damaged proteins and aggregates during cell division [61–71].

In this review, we highlight recent advances in our structural and mechanistic understanding of Hsp104. In particular, we focus on how structural changes of Hsp104 hexamers during ATP hydrolysis promote substrate remodeling and how Hsp104 deconstructs

Sup35 prions. Better structural and mechanistic understanding of Hsp104 will not only yield insight into its natural function but also open the door for directed evolution and protein design endeavors to tailor Hsp104 for specific remodeling of disease-associated aggregates and other therapeutic and research purposes.

Hsp104 Structure and Mechanism

An Hsp104 monomer is 908 residues and 102 kDa [72]. Hsp104 is composed of five domains: the N-terminal domain (NTD), two nucleotide-binding AAA+ domains (NBD1 and NBD2), a coiled-coil MD inserted within the small domain of NBD1, and a unique C-terminal extension (Fig. 1a and b) [72,73]. Like many AAA+ proteins [74], Hsp104 is only functional as a hexamer [25,75–78], but the precise structure of the ring-shaped hexamer remains unresolved and controversial [72,79–84]. Within the hexamer, each domain plays specific roles and allosterically communicates with adjacent domains and subunits [72,73]. However, the mechanistic and structural details of intraprotomer and interprotomer communication and precisely what roles the individual domains play in substrate remodeling are not fully resolved.

Crystal structures of the NTDs of the related bacterial Hsp100 proteins ClpA [85], ClpB [83,86], ClpV [87], and ClpC [88] have been solved. The NTDs are highly structurally conserved; they are very stable globular domains [85,86,88,89] made up of two imperfect repeats of four helical bundles [85,86,88] and are connected to the adjacent NBD1 by a highly mobile linker [83,90–92]. In ClpB, a bacterial Hsp104 orthologue, the NTD is involved in substrate binding [83,86,93–97,166] and casein-stimulated ATPase activity [93], and NTD mobility via the NTD–NBD1 linker is necessary for efficient translocation and disaggregation of substrate [98,99].

In contrast to the bacterial Hsp100 proteins, the function of the Hsp104 NTD has been less explored. *In vivo*, deletion of the NTD or specific point mutations (e.g., T160M) within the NTD has little effect on thermotolerance or prion propagation [30,100]. Therefore, Hsp104 NTD variants can still dissolve heat-denatured aggregates and fragment prions. However, overexpression of these mutants fails to cure [*PSI*⁺] [30,100]. We have established that the ability of Hsp104 to dissolve amyloid conformers is selectively diminished by NTD deletion [41]. Indeed, Hsp104^{ΔN} can fragment but not dissolve amyloid forms of Ure2, α -synuclein, or polyglutamine [41]. Importantly, there is a fundamental difference in how amorphous and amyloid substrates are disaggregated by Hsp104 [1,101]. To disaggregate disordered aggregates, Hsp104 subunits within the

hexamer collaborate noncooperatively via probabilistic substrate binding and ATP hydrolysis [1]. By contrast, to resolve stable amyloid, several Hsp104 subunits within the hexamer must cooperatively engage substrate and hydrolyze ATP [1]. Thus, Hsp104 hexamers display operational plasticity [1]. Deletion of the NTD impairs this plasticity, which appears to be essential for the global cooperativity needed to resolve stable amyloid [1,41]. In addition, the Hsp104 NTD also participates in substrate binding and interactions with adapter proteins such as Hsp70 [41]. Indeed, the Hsp104 NTD regulates substrate binding and prevents nonproductive competition for substrate binding by pore loops [41].

The nucleotide-binding domains (NBDs) of AAA+ proteins are highly structurally conserved, consisting of a large α/β subdomain and a small α -helical subdomain [102]. Features that define these domains include regions involved in ATP binding and hydrolysis, namely, the Walker A and Walker B motifs, the arginine finger, sensor-1 and sensor-2 motifs, as well as tyrosine-bearing pore loops that engage substrate thereby coupling conformational changes of the AAA+ protein upon ATP hydrolysis to substrate remodeling [103]. In Hsp104, NBD1 and NBD2 are not simply duplicated NBDs, but differ significantly and originate from different clades of the AAA+ superfamily [103]. NBD1 belongs to clade 3 (members include the FtsH family, p97, NSF, and katanin) and NBD2 belongs to clade 5 (members include HslU/ClpX family, RuvB, and Lon family), indicating that Hsp104 arose from a gene fusion event [103]. Each NBD is able to bind and hydrolyze ATP, but they have very different catalytic properties [104]. By fitting the steady-state kinetics of ATP hydrolysis to two independent allosteric sites, as well as subsequent mutational analyses, the general properties of the two NBDs were determined [104]. NBD1 contains a low-affinity, high-turnover site and NBD2 contains a high-affinity, low-turnover site [104]. Nucleotide binding at NBD2 is critical for Hsp104 hexamerization [75–77], which is in contrast to ClpB, where nucleotide binding at NBD1 is critical for hexamerization [90]. Thus, in this regard, Hsp104 is more similar to its mitochondrial homolog, Hsp78, which also requires nucleotide binding at NBD2 (and not NBD1) for hexamerization [105]. Furthermore, while the majority of basal ATPase activity in Hsp104 is contributed by NBD1 [104], both NBDs in ClpB contribute to its basal ATPase rate [90]. Both Hsp104 NBDs display positive cooperativity and a high degree of allosteric communication between the two (e.g., hydrolysis in NBD1 depends upon the nucleotide state of NBD2) [104]. In addition to the conserved AAA+ motifs, NBD2 contains a nuclear localization signal [106]. Indeed, Hsp104 is active in both the cytoplasm and nucleus [25,106].

Hsp104 and ClpB each contain an ~ 85 -Å-long coiled-coil MD inserted into the small α -helical subdomain of NBD1 (Fig. 1a and b) [72,83]. The MD consists of four helices that make up an antiparallel, broken coiled coil [72,83]. Helix 1 and half of helix 2 are designated motif 1, with the second half of helix 2 along with helices 3 and 4 designated motif 2 [72,83]. In ClpB, helix 3 appears to undergo conformational changes in response to nucleotide, possibly transitioning between loop and helix [83,107]. MDs of ClpB and Hsp104 are essential for disaggregation activity [90,108,109] and are the sites of interaction with Hsp70 [107,108,110–112,167]. The MD has been shown to interact with NBD1 and NBD2 in an autoinhibitory fashion, repressing activity of the hexamer [84,107,111,113,114]. Hsp70 binds directly and transiently to the MD, specifically in the region of helix 2 and 3 in motif 2 [110–112,167]. This interaction appears to relieve the autoinhibitory interactions between the MD and NBD1 [110–112,167]. Thus, it appears that the MD is a highly dynamic domain involved in regulation of hexamer activity. Indeed, point mutations in the MD can lead to inactive variants with stabilized MD–NBD1 or MD–NBD2 interactions [84,113] or hyperactive variants with enhanced unfoldase activity [7,38–40,109,113]. Interestingly, deletion of motif 1 (helix 1 and a portion of helix 2), motif 2 (a portion of helix 2, helix 3, and helix 4), or the entire MD confers a hyperactive state in ClpB [79,113]. By contrast, deletion of the MD or motif 1 inactivates Hsp104, whereas deletion of motif 2, helix 3, or helix 4 potentiates Hsp104 [109]. Thus, the requirements for Hsp104 potentiation are strikingly different from the requirements for generating hyperactive ClpB variants, indicating profound differences between Hsp104 and ClpB [1,19,109]. Regardless, many of the details of the placement, dynamics and the mechanism of MD-mediated regulation of Hsp104 and ClpB remain unresolved.

Hsp104 contains a unique C-terminal domain (CTD) of 40 residues (amino acids 869–908), which is not found in ClpB [43,72]. The region is enriched in acidic residues (42.5%), and the last four residues are a conserved DDLD motif that allows binding to the Hsp90 cochaperones Cpr7, Cns1, and Sti1 [115,116], although the importance of this interaction remains unknown as deletion of the DDLD motif does not affect thermotolerance [115]. The CTD was initially thought to be a key site of substrate interaction [117], and CTD binding to lysine-rich polypeptides stimulates ATPase activity in NBD1 via a conformational change in the MD [117]. However, subsequent work supported a model in which substrates are translocated N- to C-terminally by tyrosine-bearing pore loops [35–37], which leaves poly-Lys binding at the CTD of mysterious function. More recently, the CTD was shown to be essential for hexamerization [115,118], and though

the sequence indicates that it is highly disordered, this role implies that may not necessarily be the case. This requirement for hexamerization is surprising as the domain is absent in ClpB, indicating another difference between Hsp104 and ClpB.

Our structural and mechanistic understanding of Hsp104-mediated substrate remodeling activities remains incomplete [72,119,120]. Hsp104 assembles into dynamic ring-shaped hexamers with a central channel [1,75,81]. Hsp104 hexamers form in the presence of ADP or ATP [75–77] or in the absence of nucleotide at low salt concentrations [104]. ADP or ATP binding to NBD2 is critical for hexamerization of Hsp104, whereas ADP or ATP binding to NBD1 is dispensable for hexamerization [75–77]. Hsp104 hexamers exchange subunits on the minute timescale [1]. The hexamer undergoes large conformational changes upon ATP hydrolysis [73,82], but the details of these changes and how they are coupled to substrate remodeling remain unclear. Protein disaggregation is driven by Hsp104 coupling ATP hydrolysis to the partial or complete translocation of substrate across the central channel to solution via interaction with conserved tyrosine-bearing pore loops in NBD1 and NBD2 [33–37,41]. However, recent studies suggest that ClpB is a nonprocessive translocase, which takes only one to two translocation steps prior to releasing substrate, which appears insufficient for complete translocation across the central channel [121]. Thus, ClpB may dissociate protein aggregates by pulling and releasing exposed tails or loops [121]. In light of these studies [121], it will be important to determine the processivity of Hsp104.

Intriguingly, Hsp104 and ClpB use profoundly different mechanisms of intersubunit collaboration to disaggregate substrates. ClpB subunits couple cooperative ATP hydrolysis to probabilistic substrate binding [1]. By contrast, Hsp104 subunits display operational plasticity and can employ noncooperative substrate binding and ATP hydrolysis or cooperative substrate binding and hydrolysis depending upon the stability of the substrate [1]. Thus, more stable substrates elicit a switch in Hsp104 operating mode, such that more subunits are recruited to participate in the substrate binding and ATP hydrolysis needed for protein disaggregation [1]. This operational plasticity enables Hsp104 to adapt different mechanochemical coupling mechanisms that are tailored to the specific physical demands of the aggregated substrate [1]. Thus, if two subunits are sufficient to rapidly disaggregate a substrate, then only two will be used [1]. These differences between Hsp104 and ClpB have biological consequences. For example, ClpB has enhanced ability to dissolve disordered aggregates but extremely limited ability to dissolve amyloid [1,19,27,84]. By contrast, Hsp104 can readily dissolve both disordered aggregates and amyloid [1]. Indeed, even when ClpB is hyperactivated by mutations in the MD, it is unable to dissolve amyloid [19]. By contrast,

the equivalent mutations in Hsp104 (e.g., Y507D; Fig. 1b) potentiate the ability of Hsp104 to antagonize the aggregation and toxicity of various human neurodegenerative disease proteins, including TDP-43, FUS, or α -synuclein [7].

Interestingly, despite this limited ability to dissolve amyloid, ClpB has been reported to have a weak ability to propagate Sup35 prions under artificial circumstances in yeast [122,123]. ClpB is unable to propagate [*PSI*⁺] (Sup35 prions) in yeast [124], unless *Escherichia coli* DnaK (Hsp70) and GrpE (the DnaK nucleotide exchange factor) are also expressed [122,123]. However, even then, [*PSI*⁺] propagation is considerably weaker than usual [122], indicating that ClpB has limited ability to fragment Sup35 prions even with DnaK and GrpE. Moreover, even with DnaK and GrpE expression, ClpB was unable to propagate [*URE3*] (Ure2 prions) [123], confirming a profound difference in Hsp104 and ClpB activity against Ure2 prions [1]. It has also been reported that *E. coli* can weakly propagate amyloid forms of NM (the N and M domains of Sup35) tagged with mCherry in a manner that requires ClpB [125]. However, here too, propagation was inefficient and NM-mCherry amyloid was rapidly lost from 50% of the original *E. coli* colonies [125]. This instability stands in stark contrast to the loss of strong [*PSI*⁺] in 1 in every 10⁶ colonies in *Saccharomyces cerevisiae* [57,125]. Thus, even in its natural *E. coli* environment, the ability of ClpB to promote amyloid propagation is remarkably limited. Indeed, ClpB is not required for propagation of a RepA-WH1 prionoid in *E. coli* [126]. Finally, it is important to note that prion propagation in yeast can be driven solely by prion fragmentation and does not require prion dissolution per se [41,100,127,128]. Indeed, prion fragmentation and dissolution are separable phenomena [41]. For example, Hsp104 ^{Δ N} can fragment Sup35 prions without releasing soluble Sup35 and is unable to dissolve Sup35 prions and other amyloids [41]. We suggest that, in the presence of DnaK, DnaJ, and GrpE, ClpB has a very weak ability to fragment some amyloids [122,123,125] but not others [123,126]. Indeed, even very low activity of select Hsp104 mutants can propagate unstable prion variants in yeast [129,130]. Nevertheless, ClpB has diminished ability to dissolve amyloid in comparison to Hsp104, which stems from an altered mechanism of intersubunit collaboration [1,19,27,84].

The hexameric structure of Hsp104 remains unresolved. In fact, two radically distinct models were initially advanced [72]. Both were based on cryo-electron microscopy (cryo-EM) reconstructions with rigid-body fit domains from the crystal structure of the *Thermus thermophilus* homolog, tClpB [83]. Unfortunately, tClpB was not resolved in the functional, hexameric state [83]. Using assumptions based on other AAA+ proteins, an initial hexameric

structure with an external placement of the coiled-coil MD was proposed [83]. However, this structure was incompatible with the dimensions of Hsp104 hexamers observed by another cryo-EM study [81]. This incompatibility led to a second model with the coiled-coil MD intercalated between the two nucleotide-binding domains [81,82]. However, this new model did not preserve the typical intersubunit AAA+ nucleotide-binding sites, and the debate over the hexameric structure has continued [72,131].

More recently, a revised cryo-EM model has been proposed suggesting that the coiled-coil MD is on the surface of the hexamer, but can adopt diverse horizontally tilted positions [79]. This new placement of the MD preserves more canonical AAA+ architecture and is supported by site-resolved fluorescence, site-resolved cross-linking, site-resolved biotin labeling, and hydrogen–deuterium exchange studies [79,113]. Moreover, x-ray footprinting, site-resolved cross-linking, and site-resolved fluorescence studies reveal that the Hsp104 MD cannot project out into solution as initially proposed [80,83], but rather, it adopts a position that contacts NBD2 [84]. Thus, it appears that the MD can adopt diverse positions on the exterior of the hexamer [79,84,113].

Conformational Changes of Hsp104 Hexamers during ATP Hydrolysis

Hsp104 is a large, dynamic, multidomain hexamer that couples large conformational changes upon ATP hydrolysis to exertion of mechanical force on myriad substrates [73]. However, the nature of these conformational changes and how they promote substrate remodeling have remained unclear. To address this issue and enable an independent and complementary view, we have utilized small-angle x-ray scattering (SAXS) and wide-angle x-ray scattering (WAXS), which has provided important insights into the conformational changes of several other AAA+ proteins [132–136] and complex chaperonins [137] in solution. In previous SAXS studies of AAA+ proteins, crystal structures of domains fit well into the *ab initio* volumes [132–135], which allowed for hexameric models to be built from monomeric crystal structures and changes in conformation to be modeled based on the volumetric reconstructions. Additionally, changes in physical properties such as R_g and D_{max} and conformational changes of the reconstructed volumes in the presence of different nucleotides allowed for novel mechanistic understanding of wild-type and mutant proteins [132,133,135]. Cryo-EM reconstructions of Hsp104 have not yet yielded a unified picture of how Hsp104 catalyzes protein disaggregation [72,79–82,131]. Moreover, only dysfunctional variants of Hsp104 (e.g., HAP plus ClpP, Hsp104^{N728A},

Hsp104^{ΔN}, and Hsp104^{E285A:E687A}) have been studied in a limited number of nucleotide states (only ATPγS, ATP, and ADP have been explored) [79–82]. Consequently, it remains difficult to extrapolate these findings to understand wild-type Hsp104. Importantly, SAXS/WAXS is performed in solution, under conditions where Hsp104 is active. Indeed, SAXS has emerged as powerful method for visualizing the workings of biological machines in solution [138] and avoids any potential issues caused by freezing or fixation in cryo-EM.

In this context, we were very interested to compare Hsp104 to Hsp104^{ΔN}, as Hsp104^{ΔN} is a hypomorphic disaggregase that is unable to dissolve amyloid conformers but can dissolve disordered aggregates [41]. Thus, we examined Hsp104 and Hsp104^{ΔN} in AMP-PNP (a nonhydrolyzable ATP analog), ATPγS (a slowly hydrolyzable ATP analog), ATP, ADP-AIF_x (hydrolysis transition-state mimic), ADP, and apo states by SAXS/WAXS to a nominal resolution limit of ~7.6–8.4 Å [41]. By studying Hsp104 in these various nucleotide states, we can elucidate hexameric states that are likely to be populated during its natural reaction cycle [41]. Volumetric reconstructions reveal Hsp104 and Hsp104^{ΔN} hexamers that can readily accommodate six monomers via rudimentary domain fitting (Fig. 2) [41]. Moreover, Hsp104 and Hsp104^{ΔN} hexamers exhibit large structural differences between these different nucleotide conditions (Fig. 3a and b) [41]. Indeed, our findings indicate that Hsp104 and Hsp104^{ΔN} hexamers contract upon ATP hydrolysis and expand upon ATP binding, suggesting a pumping mechanism to drive substrate translocation [41]. Interestingly, SAXS reconstructions of another AAA+ protein, p97, revealed that the p97 hexamer also appears to contract upon ATP hydrolysis (ADP-AIF_x state) [133]. Thus, cycles of hexamer expansion and contraction coupled to ATP binding and hydrolysis could be a general feature of how certain AAA+ proteins remodel substrates.

One striking feature of the Hsp104 and Hsp104^{ΔN} hexamers was the positioning of a projection of density on the hexamer exterior, along the plane of the largest dimension [41]. Hsp104 and Hsp104^{ΔN} have external projections that occupy a more N-terminal position (in no nucleotide, AMP-PNP, ATPγS, and ATP) to a more C-terminal position (in ADP-AIF_x and ADP) upon ATP hydrolysis (Fig. 3a and b, arrows) [41]. It is probable that the external projection corresponds to the MD [41], which is consistent with cryo-EM reconstructions of HAP plus ATPγS and ClpP [79]. The SAXS reconstruction of Hsp104 in ATPγS and cryo-EM reconstruction of HAP plus ATPγS and ClpP reveal particles of similar dimensions (Fig. 4) [41,79]. We suggest that our SAXS reconstructions help to clarify MD location [41,79–82,131]. The shift

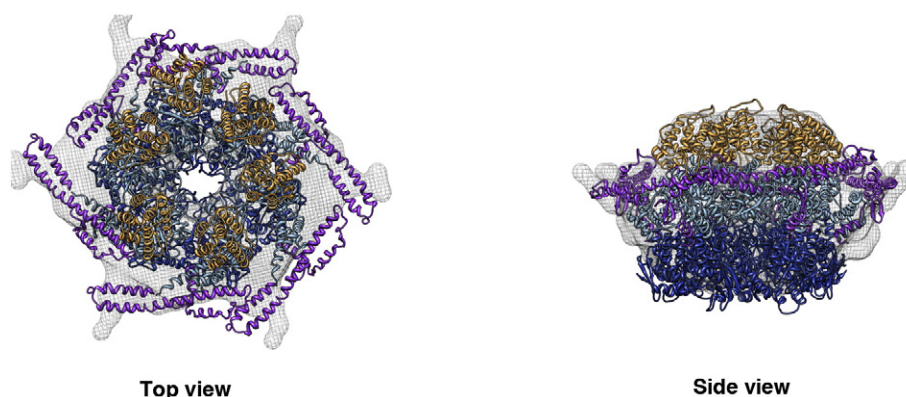


Fig. 2. Rigid-body fitting individual Hsp104 domains into SAXS reconstructions of the Hsp104 hexamer in the presence of ATP. Individual Hsp104 domains were homology modeled based on the ClpB crystal structure PDB ID: 1khy (NTD) and PDB ID: 1qvr (for NBD1, MD, and NBD2). The domains were then rigid-body fit into the volume envelope generated from SAXS/WAXS data of the Hsp104 hexamer in the presence of ATP. Top and side views are shown. The individual domains are color coded: NTD (orange), NBD1 (light blue), MD (purple), and NBD2 (dark blue). Adapted from Ref. [41].

in positioning of the external projection (Fig. 3a and b, arrows) suggests that the MD might move from an N-terminal horizontal position to a C-terminally tilted position upon ATP hydrolysis [41,79,84].

To disaggregate substrates, Hsp104 is thought to translocate proteins either partially or completely through its central channel [19,33,37]. Additional striking features of Hsp104 and Hsp104^{ΔN} hexamers were changes in the diameter and contours of the central channel in the various nucleotide states (Fig. 5a and b) [41]. The motions of the Hsp104 channel are highly reminiscent of a peristaltic wave [41]. In a peristaltic wave, there is a relaxation at the site of substrate entrance, followed by a wave of constriction that travels in the direction the substrate is being pumped. Substrate enters through the N-terminal opening and can be fully translocated out the C-terminal end of the channel [19,37]. In the ATP_γS or ATP states, when the hexamer is primed to engage substrate, the extreme N-terminal side is open (Fig. 5a). After the opening, there is a region of constriction, still N-terminal (Fig. 5a, arrow). As we step through the simulated ATPase cycle, the channel first constricts fully, correlating with the most contracted state, in the ADP-AIF_x transition-state mimic, and then the point of constriction moves toward the C-terminus in the ADP state (Fig. 5a). This peristaltic pumping motion explains how the Hsp104 hexamer is able to transduce energy from ATP hydrolysis to conformational change and substrate remodeling using physical force.

The role of the NTD in facilitating the peristaltic pump motion is both simple and significant. In the absence of the NTD, the channel is unable to contract; rather, the entire hexamer is smaller in the N- to C-terminal direction, and the channel appears

to have a uniformly large diameter throughout (Fig. 5b). As the Hsp104^{ΔN} channel progresses through the ATPase cycle, it is clear that the missing domain is essential for the proper movements of the channel. This defect is most obvious in the transition-state mimic ADP-AIF_x, which is constricted in the center but open on both sides (Fig. 5b). While there still appears to be an area of contraction that shifts N- to C-terminally, it is clear that the peristaltic motion is greatly perturbed in the Hsp104^{ΔN} hexamer (Fig. 5b). Specifically, it appears that substrate could more readily diffuse out of the N-terminal opening of the channel of the Hsp104^{ΔN} hexamer.

Our SAXS findings indicate that deletion of the NTD results in expansion of the ends of the central cavity (similar to a cryo-EM study of the Hsp104^{ΔN} hexamer [81]), as well as abnormal changes of the central channel during the ATPase cycle (Fig. 5b). Hsp104^{ΔN} is unable to undergo nucleotide-dependent changes in the central channel similar to Hsp104, which helps explain the reduced ability of Hsp104^{ΔN} to translocate substrate through the central channel [41].

How Hsp104 Deconstructs Sup35 Prions

We have also recently elucidated the mechanism of Sup35 prion fragmentation and dissolution by Hsp104 [41]. Sup35 is an essential translation termination factor that can switch to various prion conformations that encode [PSI⁺] phenotypes [57]. Sup35 contains a C-terminal GTPase domain (amino acids 254–685, black), a highly charged MD (M, amino acids 124–253, dark gray), and a

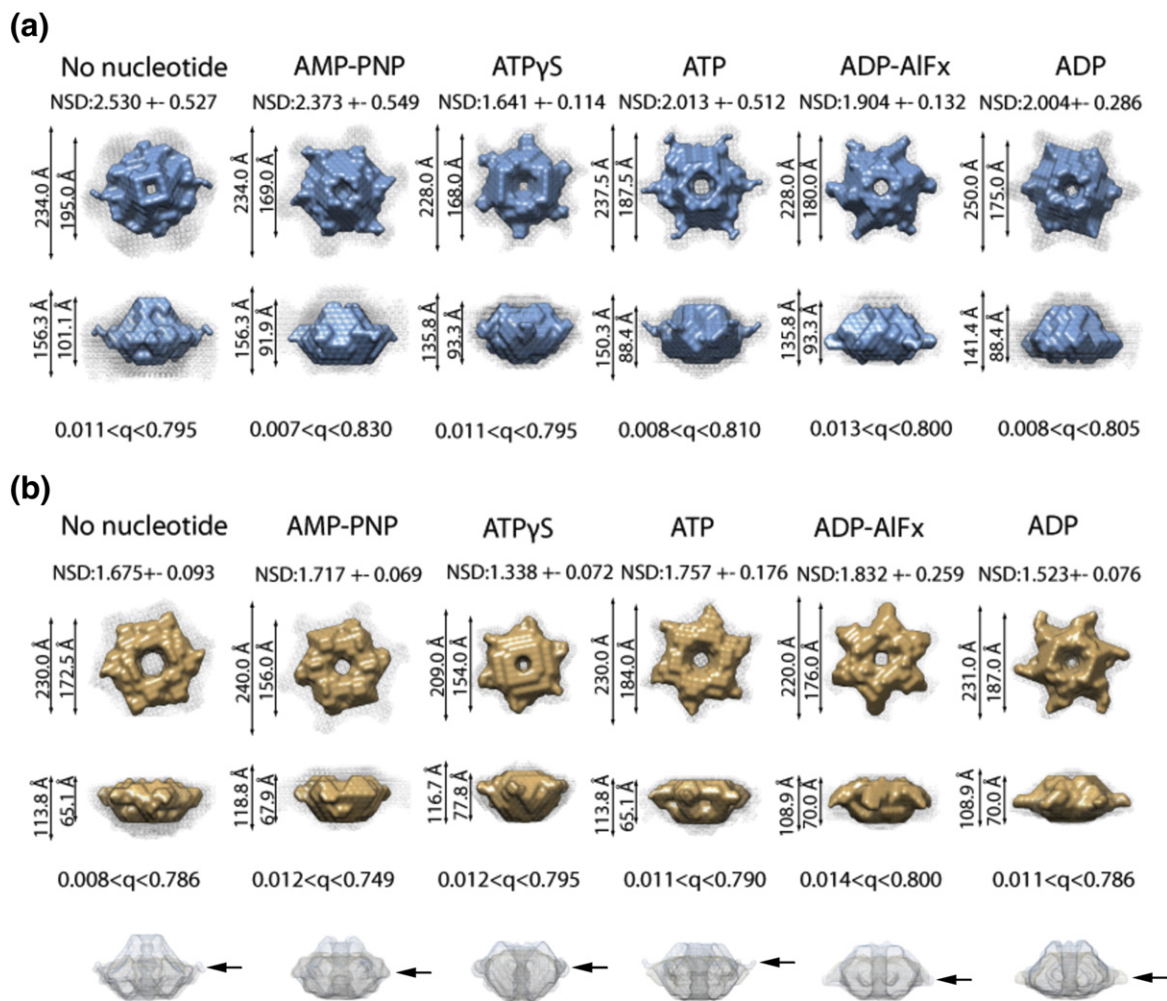


Fig. 3. NTD deletion alters ATPase-driven conformational changes of Hsp104 hexamers. Volumetric envelope reconstructions of Hsp104 and Hsp104 Δ N derived from SAXS/WAXS data. Averaged *ab initio* GASBOR [164] volume reconstructions of Hsp104 (a) and Hsp104 Δ N (b) from the SAXS/WAXS data. Filtered density is filled blue (Hsp104) or orange (Hsp104 Δ N) overlaid with the unfiltered average shown in gray mesh. Normalized spatial discrepancy (NSD) of the averaged models and the q range used for the reconstructions are shown along with the average particle dimensions. Overlay of the Hsp104 and Hsp104 Δ N average reconstructions for each state, which were used to orient the particles, is shown at the bottom of (b). The Hsp104 and Hsp104 Δ N hexamers undergo large changes in shape that depend upon the identity of the nucleotide. Reconstructions are oriented with the N-terminus pointing toward the top of the page. Arrows denote position of external projection, likely corresponding to the MD, in each state. Adapted from Ref. [41].

prionogenic NTD (N, amino acids 1–123, light gray) enriched in glutamine, asparagine, tyrosine, and glycine residues (Fig. 6a) [57]. Within N, prion recognition elements establish homotypic intermolecular contacts such that alternating head-to-head (red) and tail-to-tail (green) contacts hold adjacent Sup35 monomers together within the assembled prion (Fig. 6a) [41,139–146]. A central core (blue) in between these prion recognition elements is sequestered by intramolecular cross- β contacts (Fig. 6a) [41,139–146]. The residues that comprise the head, the central core, and the tail vary depending on the Sup35 prion strain that assembles [41,139–146].

Using site-resolved cross-linking, we have established that Hsp104 initially engages assembled Sup35 prions by binding to a region spanning amino acids 96–151 of the N and M domains (Fig. 6b, purple regions) [41]. This observation is consistent with previous peptide array binding studies [147]. After this initial engagement, Hsp104 then exerts a directed pulling force that selectively unfolds cross- β structure N-terminal to this initial binding site (Fig. 6b) [41]. Remarkably, Hsp104 does not unfold regions C-terminal to this binding site (Fig. 6b) [41]. This specific partial translocation mechanism enables Hsp104 to dissolve Sup35 prions without unfolding the C-terminal

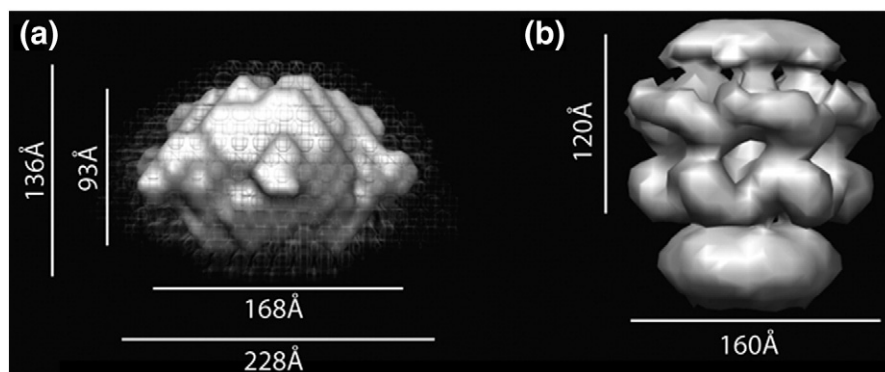


Fig. 4. SAXS reconstruction of Hsp104 in ATP γ S and the cryo-EM reconstruction of HAP plus ClpP in ATP γ S. (a) Side view of SAXS reconstruction of Hsp104 in ATP γ S. Filtered (filled) and unfiltered density (caged) plus dimensions are indicated [41]. (b) Cryo-EM reconstruction of HAP plus ClpP in ATP γ S [79]. Dimensions are indicated. Note the density of ClpP below the HAP envelope.

GTPase domain (Fig. 6b) [41]. Thus, Hsp104 rapidly releases functional, folded Sup35 from the assembled prion and can rapidly eliminate the

loss-of-function [*PSI*⁺] prion phenotype [27,30,32,148,149]. This type of partial translocation mechanism may also enable Hsp104 to rapidly

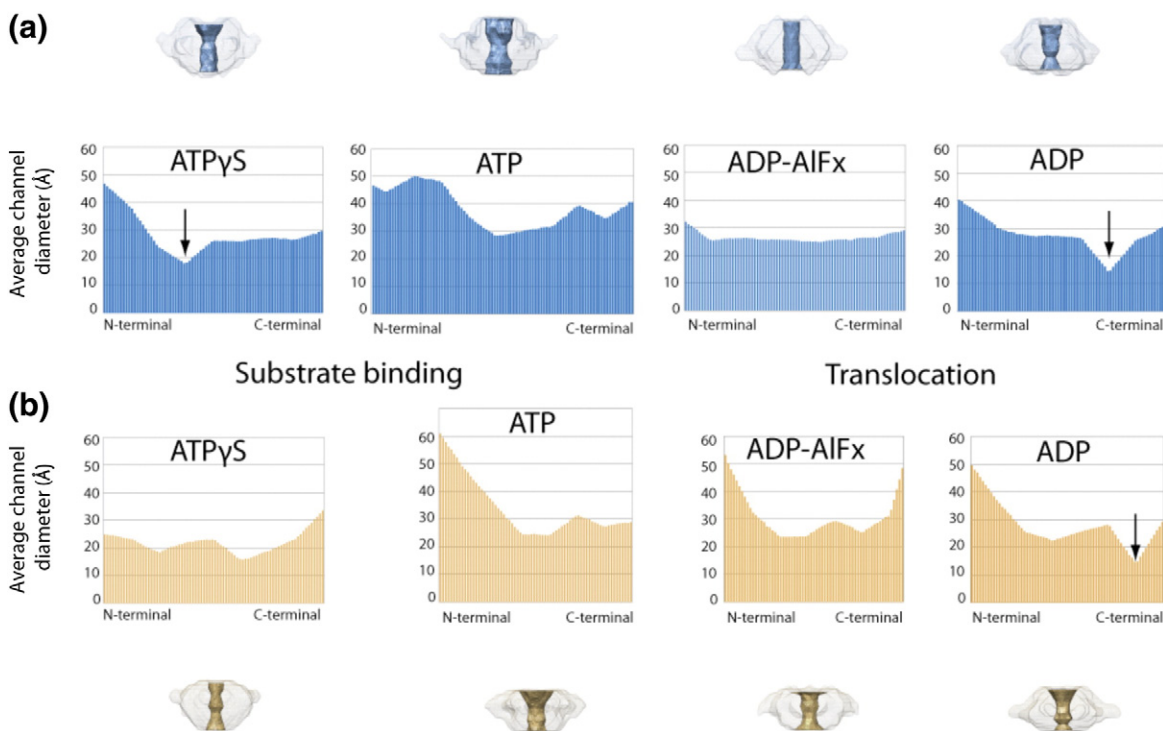


Fig. 5. Channel motions of the Hsp104 and Hsp104 Δ N hexamers. (a and b) Mapping of the Hsp104 (a) and Hsp104 Δ N (b) hexamer channels. Cut-away side views of the Hsp104 (a) and Hsp104 Δ N (b) hexamers in the ATP γ S, ATP, ADP-AIF $_x$, and ADP state are shown. The volume of the channel was reconstructed in Chimera [165] using the filtered average volumes for each nucleotide state. The bar graphs display the average diameter of each 1 Å z slice of the channel volume starting from the N-terminus as calculated using Matlab. Substrate binds in the ATP γ S state of the hexamer and is translocated from the N-terminal entrance to the C-terminal exit. The Hsp104 channel exhibits a peristaltic wave motion; relaxation at the N-terminal entrance followed by a contraction of the N-terminal end of the channel and finally a shift in the location of a constriction from the N-terminal to the C-terminal region (arrow). The Hsp104 Δ N hexamer displays defects in the peristalsis motion, especially in the N-terminal entrance to the channel, which fails to contract in the ADP-AIF $_x$ and ADP states. This defect is most noticeable in the ADP-AIF $_x$ state. Adapted from Ref. [41].

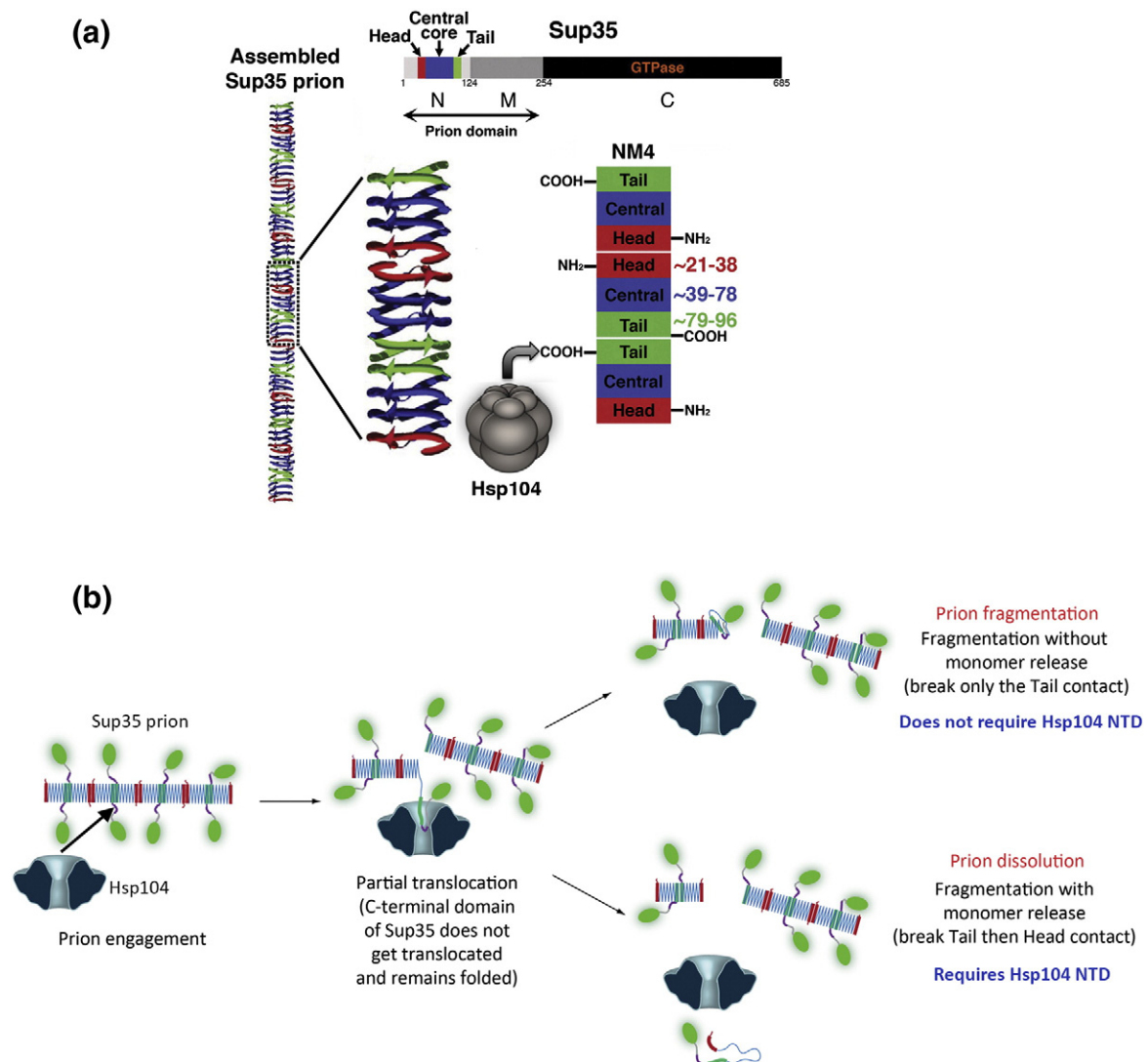


Fig. 6. How Hsp104 deconstructs Sup35 prions. (a) Sup35 is composed of a C-terminal GTPase domain (amino acids 254–685, black) that confers translation termination activity, a highly charged MD (M, amino acids 124–253, dark gray), and a prionogenic NTD (N, amino acids 1–123, light gray) enriched in glutamine, asparagine, tyrosine, and glycine. Within N, prion recognition elements within the NTD of Sup35 make homotypic intermolecular contacts such that Sup35 prions are maintained by an alternating sequence of head-to-head (red) and tail-to-tail (green) contacts. The central core (blue) is sequestered by intramolecular contacts. The position of the head contact, central core, and tail contact is shown for NM4 prions. Hsp104 is depicted engaging Sup35 prions C-terminal to the tail contact. (b) Model of Sup35 prion fragmentation *versus* dissolution by Hsp104. Hsp104 initially engages Sup35 prions in a region (amino acids 96–151; purple) C-terminal to the tail contact (dark green). Directional pulling on the N-terminal cross- β structure leads to partial translocation and breakage of the tail contact and Sup35 prion fragmentation. Further translocation breaks central core contacts (blue) and the head contacts (red) resulting in monomer release. In this way, the Sup35 prion is fragmented with or without monomer release, and the CTD of Sup35 remains folded throughout this process. Hsp104 ^{Δ N} retains the ability to break the tail but not the central core or head contacts. Therefore, Hsp104 ^{Δ N} is able to fragment Sup35 prions but not resolubilize Sup35 monomers. Adapted from Ref. [41].

disassemble heat-induced assemblies of functional proteins and immediately release active, soluble protein [33,60]. By contrast, remodeling of seminal amyloid fibrils formed by PAP248-286 and PAP85-120 appears to involve complete translocat-

tion of the peptides across the Hsp104 channel [19] as with other model substrates [37]. Thus, Hsp104 can exhibit plasticity in the translocation mechanism employed to resolve different aggregated structures.

After binding to the Sup35 prion, Hsp104 selectively remodels cross- β structure N-terminal to its binding site in three steps [41]:

- (1) Hsp104 severs the tail-to-tail contact to fragment the prion (Fig. 6b, dark-green regions) [41].
- (2) Hsp104 unfolds the central cross- β core (Fig. 6b, blue regions) [41].
- (3) Hsp104 breaks the head-to-head contact (Fig. 6b, red regions) to release a soluble, folded Sup35 monomer [41].

Intriguingly, Hsp104 ^{Δ N} is dysfunctional in the second and third steps of this stepwise prion-dissolution process [41]. Hsp104 ^{Δ N} binds assembled Sup35 prions at the same site as Hsp104 and proceeds to rupture the tail-to-tail contact [41]. However, Hsp104 ^{Δ N} is subsequently unable to remodel the central core or separate the head-to-head contact [41]. Thus, Hsp104 ^{Δ N} is a hypomorphic disaggregase that fragments but does not dissolve Sup35 prions (Fig. 6b) [41]. This finding helps explain why Hsp104 ^{Δ N} can propagate [PSI⁺] but is much less able to cure [PSI⁺] at high concentrations *in vivo* [30,100]. Indeed, deleting the NTD restricts Hsp104 activity to a mode where it preferentially stimulates Sup35 prionogenesis and amplifies prions that confer strong [PSI⁺] [41,145].

Several *in vivo* studies suggest that elevated Hsp104 concentrations can cure yeast of [PSI⁺] (Sup35 prions) via direct dissolution of Sup35 prions by Hsp104 [29,30,148,150]. It has also been suggested that Sup35 prion remodeling by excess Hsp104 yields noninfectious Sup35 amyloid-like conformers with reduced seeding activity [32,145,147,151]. Indeed, remodeling of pure Sup35 prions by Hsp104 yields a mixture of soluble Sup35 and nontemplating, amyloid-like Sup35 *in vitro* [31,32,145]. Moreover, overexpression of Hsp104 in yeast cures [PSI⁺] and yields a mixture of soluble Sup35 and SDS-resistant, amyloid-like forms of Sup35 [152]. However, mechanisms distinct from prion remodeling or dissolution have been proposed to explain [PSI⁺] curing by Hsp104 overexpression. For example, it has been proposed that an indirect inhibition of Sup35 prion fragmentation due to displacement of Ssa1 from Sup35 prions by excess Hsp104 cures [PSI⁺] [153]. However, these experiments were performed exclusively with fluorescently tagged proteins and remain uncorroborated with native, untagged proteins and native prions *in vivo* [153]. Indeed, it remains unclear if the fluorescence and colocalization techniques employed in this study truly measure prion fragmentation or even direct prion-chaperone binding events [153]. Finally, it is important to note that

[PSI⁺] curing kinetics by Hsp104 overexpression are simply too rapid to be explained solely by an inhibition of prion fragmentation [30,154].

Essential Role for the NTD in Potentiated Hsp104 Activity

We have established that the NTD is essential for potentiation of Hsp104 activity by mutations in the MD [41]. Unlike their full-length equivalents, neither Hsp104 ^{Δ N-A503V} nor Hsp104 ^{Δ N-A503S} rescued TDP-43, FUS, or α -synuclein aggregation or toxicity in yeast [41]. Potentiating mutations at the A503 position of the MD (Fig. 1b) likely destabilize a fragilely constrained autoinhibited state that restricts Hsp104 activity or mimic a critical allosteric activation step [7,38–40]. Consequently, these mutations enhance Hsp104 ATPase activity, substrate translocation rate, unfoldase activity, and disaggregase activity [7]. These hyperactivating sequelae are nixed by NTD deletion [41], which suggests that future designs of potentiated Hsp104 variants should include the NTD. Further studies will clarify any direct role for the NTD in potentiated Hsp104 activity. However, potentiation may simply require the critical role of the NTD in nucleotide-dependent conformational changes that enable productive hexamer cooperativity [41]. It will be interesting to determine whether mutations in the NTD can potentiate Hsp104 activity in the absence of MD mutations or whether novel NTD mutations can further enhance the activity of potentiated Hsp104 variants.

Closing Remarks

Our recent advances highlight the importance of the Hsp104 NTD in enabling the full repertoire of Hsp104 activities [41], whereas previously, the NTD has been considered to be more dispensable [35,90,94,100,153]. Indeed, the Hsp104 NTD enables globally cooperative substrate translocation, which is critical for prion dissolution and potentiated disaggregase activity [41]. In other AAA+ proteins, such as Lon, the NTD is more stringently integral to enzyme function [155]. By contrast, other AAA+ proteins harbor NTDs that can appear at least partially dispensable as with ClpA or ClpX [89,156–159] or even negatively regulate activity, as with Cdc48 and PAN [160–163]. However, we suggest that optimal functionality for ring-shaped, hexameric AAA+ proteins that must unfold structurally diverse repertoires of misfolded proteins or disrupt divergent protein complexes depends on NTDs that enable hexamer plasticity and potentiation [41].

Acknowledgements

We thank Dr. Meredith Jackrel, Korrie Mack, and Zachary March for comments on the manuscript. Our work was funded by American Heart Association predoctoral fellowship (E.A.S.); National Institutes of Health (NIH) training grant (T32GM008275; E.A.S.); and NIH Director's New Innovator Award (DP2OD002177), Ellison Medical Foundation New Scholar in Aging Award, Target ALS, a Muscular Dystrophy Association Research Award (MDA277268), the Robert Packard Center for ALS Research at Johns Hopkins University, and NIH grant R01GM099836 (J.S.).

Received 18 September 2015;

Received in revised form 11 November 2015;

Accepted 12 November 2015

Available online xxxx

Keywords:

Hsp104;
prion;
disaggregase

Abbreviations used:

NTD, N-terminal domain; MD, middle domain; CTD, C-terminal domain; cryo-EM, cryo-electron microscopy; SAXS, small-angle x-ray scattering; WAXS, wide-angle x-ray scattering; NIH, National Institutes of Health; NBD, nucleotide-binding domain.

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