Navigating the ClpB channel to solution

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New work places the Hsp70/DnaK system in an opening role in protein disaggregation, facilitating the extraction of individual polypeptides from the aggregate surface to the axial channel of Hsp104/ClpB. It also reinforces a long-standing belief that reactivation rather than simple clearance of aggregated protein is necessary for cell stress tolerance.

Excessive protein misfolding and aggregation can be fatal, and contribute to devastating disorders as diverse as sickle cell anemia, type II diabetes and new variant Creutzfeldt-Jakob disease[1]. However, to function optimally, cells must maintain a highly crowded macromolecular interior (up to 400 mg ml^{-1}), which accentuates the risk of protein aggregation[2]. Thus, sophisticated molecular chaperone, osmolyte and proteolytic systems have evolved to minimize protein misfolding and eliminate misfolded conformers when they appear[1,2]. Environmental stress, such as high temperatures, can overwhelm these systems and induce potentially catastrophic protein aggregation. In fungi, the survival of such insults is increased up to 10,000-fold by Hsp104 (refs. 3,4), a member the Hsp100/Clp subfamily of AAA+ (ATPases associated with diverse activities) proteins. The Hsp104 homologs ClpB and Hsp101 are equally critical in stress tolerance in bacteria[5] and plants[6]. Hsp104/ClpB collaborate with the Hsp70/DnaK system to dissolve and reactivate aggregated proteins[4,7,8]. Protein aggregates are notoriously irksome to study and precisely how their dissolution is achieved remains unclear. Elegant studies by Mogk and colleagues[9] now illuminate several mechanistic aspects of ClpB-mediated disaggregation and its consequences for cell viability after heat shock.

Hsp100/Clp proteins, like other AAA+ proteins, couple ATPase activity to substrate unfolding and/or disruption of macromolecular complexes[10]. Like other Hsp100/Clp proteins, Hsp104/ClpB forms barrel-shaped hexamers in the presence of ATP, with each protomer constituting one stave of the barrel[11] (**Fig. 1**). Each ClpB protomer is composed of an N-terminal domain and two AAA+ modules (AAA-1 and AAA-2) that are separated by a coiled-coil middle domain (**Fig. 1a**). An axial channel wide enough (~10–16 Å in diameter) to conduct linearized polypeptides spans the length of ClpB hexamers (**Fig. 1b**,**c**) while the coiled-coil middle domain protrudes from the hexamer exterior resembling a two-bladed propeller[11] (**Fig. 1b**). Cooperative ATPase activity at both AAA-1 and AAA-2 as well as allosteric communication between AAA-1 and AAA-2 coordinated by substrate binding are critical for Hsp104 function[12,13]. ATP hydrolysis induces large motions of the Hsp104/ClpB hexamer[11,13], which, coupled to substrate interactions, provide the critical forces that drive disaggregation. It remains unclear precisely how this is mediated by the specialized architecture of Hsp104/ClpB hexamers and how Hsp104/ClpB interfaces with the Hsp70/DnaK system.

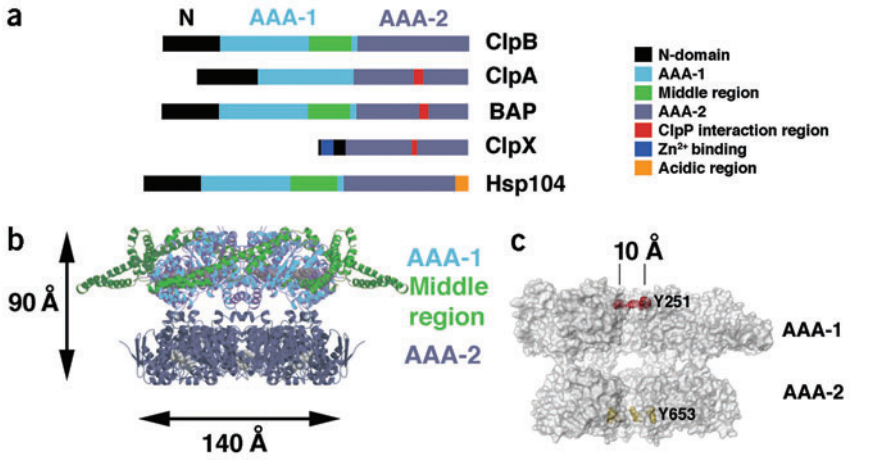

Figure 1 The architecture of the ClpB hexamer. (**a**) Domain organization of ClpB, ClpA, BAP, ClpX and Hsp104. Note the addition of the ClpP-interacting helix-loop-helix motif of ClpA to ClpB to generate BAP. Hsp104 is organized similarly to ClpB, but contains an additional C-terminal acidic domain. (**b**) Hexameric model of ClpB lacking the N-terminal domain. The side view shows the AAA-1 (light blue) and AAA-2 domains (purple). Note the propeller-like projections of the coiled coil middle domain (green). AMP-PNP molecules are denoted as CPK models (gray)[9]. (**c**) Positions of Tyr251 (red) and Tyr653 (yellow) are denoted as CPK models in this side-view surface representation of the ClpB hexamer lacking the N-terminal domains and with two monomers removed[9].

To deconstruct the mechanism of ClpB-driven disaggregation, Weibezahn *et al.*[9] engineered ClpB to be more akin to two related Hsp100/Clp proteins, ClpX and ClpA (**Fig. 1a**). ClpX and ClpA unfold and deliver appropriate substrates via their axial channel to the precisely aligned entry portal of the compartmental peptidase ClpP for degradation[10]. Interaction of ClpX and ClpA with ClpP is mediated by a short IGF/L loop that resides in a helix-loop-helix motif of their AAA-2 domain and is not conserved in ClpB[9,10] (**Fig. 1a**,**b**). An engineered ClpB containing the IGL loop of ClpA, termed BAP (ClpB-ClpA P-loop) (**Fig. 1a**), behaved similarly to ClpB *in vitro* and *in vivo*, but also associated with and delivered substrates to ClpP for degradation[9]. BAP-ClpP degraded aggregated substrates in a manner that depended absolutely on the DnaK system (DnaK, DnaJ and GrpE, termed KJE)[9]. Disaggregation and degradation were kinetically coupled as disaggregated substrates were passed rapidly from BAP to ClpP (but not to a defective ClpP variant) and were inaccessible to KJE or added GroEL[9]. Because ClpX and ClpA deliver substrates to the ClpP digestion chamber via their axial channel[10], it was suggested that, by analogy, KJE acts first on aggregates and allows ClpB to unravel and translocate polypeptides from the aggregate surface to solution by passage through its axial channel[9] (**Fig. 2a**).

The placement of KJE activity prior to BAP in disaggregation[9] and other kinetic studies[7,14] would seem to contradict previous indications that the Hsp70/DnaK system acts after Hsp104/ClpB in disaggregation[8,15]. However, Hsp70/DnaK may still be required to capture

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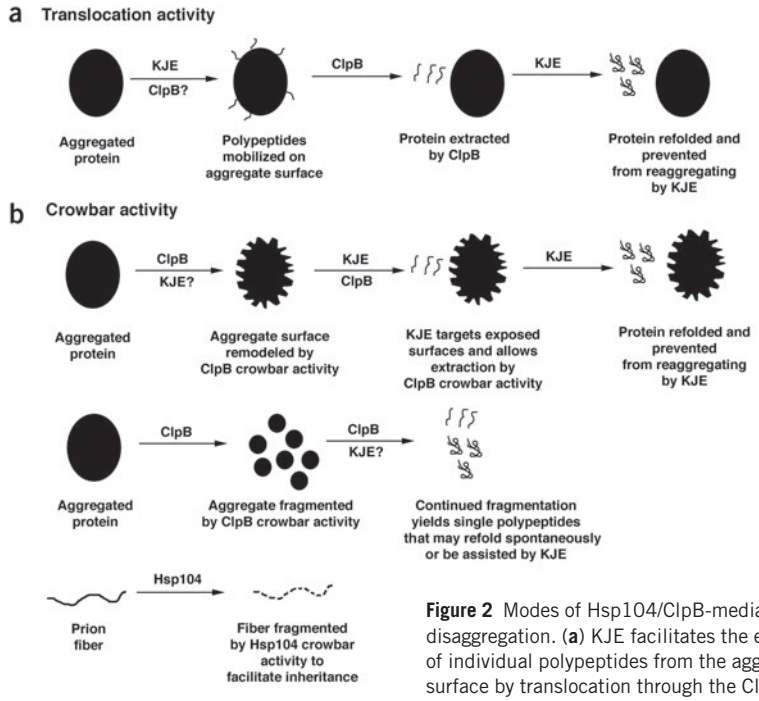

Figure 2 Modes of Hsp104/ClpB-mediated disaggregation. (**a**) KJE facilitates the extraction of individual polypeptides from the aggregate surface by translocation through the ClpB pore. Extracted protein is then refolded and prevented from reaggregating by KJE. (**b**) A 'crowbar' activity of ClpB may remodel the aggregate surface to allow KJE binding, which, accompanied by further crowbar activity, may liberate individual polypeptides that are then refolded by KJE. Alternatively, ClpB crowbar activity may fragment larger aggregates into smaller pieces until single polypeptides are released that may refold spontaneously or be assisted by KJE. Such an activity may be most likely to fragment linear polymers, such as the prion fibers fragmented by Hsp104. These models need not be mutually exclusive and may operate sequentially or simultaneously in a substrate-dependent manner.

newly disaggregated, unfolded polypeptides once they are released by Hsp104/ClpB[7,16]. The importance of this step cannot be assessed in the BAP-ClpP system because it is eliminated by substrate proteolysis[9]. However, as ClpP can degrade only unfolded substrates[10], BAP must release unfolded substrates. Thus, Hsp104/ClpB may also release substrates in an unfolded state. Whether the Hsp70/DnaK system is required to complete substrate reactivation at this stage depends on the inherent capacity of individual substrates to refold spontaneously in the crowded environment of the cell (**Fig. 2b**).

If the axial channel of Hsp100/ClpB participates in disaggregation, then residues that project into the channel might contact substrates and contribute to Hsp104/ClpB function. Two highly conserved tyrosines (Tyr251 and Tyr653 in ClpB) located in each AAA+ module project into the pore close to the entrance of either end of the ClpB hexamer[9,17] (**Fig. 1c**). Tyr653 corresponds to the tyrosine in the conserved, flexible GYVG loop of HslU (another Hsp100/Clp family member), which may control channel aperture in response to nucleotide binding[18]. Mutation of either of these tyrosines to alanine abrogates Hsp104/ClpB function in thermotolerance and disaggregation without grossly affecting hexamer integrity or ATPase activity[9,17,19]. Importantly, ClpB Tyr251 and Tyr653 contact substrates directly as revealed by crosslinking studies in which either Tyr251 or Tyr653 was replaced with a photocrosslinkable amino acid, *p*-benzoyl-L-phenylalanine[9,17]. Because these residues are near the entrances of the ClpB channel[9] (**Fig. 1c**), it is not clear that substrates are traversing the channel. Although direct demonstration of disaggregating substrate encapsulated in the ClpB channel is lacking, taken together with the KJE/BAP/ClpP-mediated degradation of aggregated protein, these data argue that disaggregation proceeds as polypeptides are conducted through the ClpB channel along an N-terminal to C-terminal vector as in ClpA or ClpX[9,10,17].

A concern with extrapolating from these beautiful studies[9] to a general mechanism for all substrates is that ClpP may profoundly alter the behavior of BAP hexamers relative to ClpB hexamers. For example, communication between ClpX and ClpP mediated by the IGF loop of ClpX inhibits ClpX ATPase activity during substrate processing and may even facilitate substrate transfer from ClpX to ClpP by coordinating alterations in the aperture of the ClpX axial channel and the ClpP entry portal[10,20]. ClpP can even rescue unfolding defects of certain ClpX mutants[20]. Whether ClpP imposes similar effects on BAP that do not ordinarily occur in ClpB remains unclear.

The function of the large coiled-coil middle domain of ClpB in threading polypeptides through the axial channel also remains a mystery. This domain is absent in ClpA and ClpX, and so would seem dispensable for transport across the channel. Yet it is critical for Hsp104/ClpB function[13,21,22]. Intriguingly, unlike ClpB or BAP, ClpA can disaggregate substrates without KJE[23], which might suggest that KJE cooperates with the middle region of ClpB. In Hsp104, polylysine engagement by the C-terminal domain elicits ATP hydrolysis by AAA-2, followed by a large conformational change in the middle domain and ATP hydrolysis by AAA-1 (ref. 13). Further, mobility of the middle region is critical for ClpB-mediated disaggregation[11]. It may be that by anchoring to protein aggregates via its C-terminal domain, or perhaps even by interactions between substrate and pore tyrosines, Hsp104/ClpB can apply leverage to other regions of the aggregate through the coordinated large motions of the coiled-coil region[11,13]. Depending on the underlying architecture of specific aggregates, this 'crowbar' activity[7] may fragment aggregates (**Fig. 2b**), similarly to the fragmentation of Sup35 prion fibers by Hsp104 (ref. 24) (although extraction of individual Sup35 molecules that link adjacent Sup35 molecules might also fragment fibers). This does not require Hsp70 and is critical in exposing new surfaces for conformational replication and prion inheritance[24]. However, in the context of large, thermally denatured aggregates, such shearing may[8] or may not[9,17] yield smaller aggregates. Instead, shearing might remodel the aggregate to expose hydrophobic surfaces that would be targeted by KJE, permitting threading through the ClpB channel (**Fig. 2a**) or helping to release substrates by continued ClpB crowbar activity (**Fig. 2b**).

Switching ClpB activity from a refolding disaggregase to a degrading disaggregase allowed assessment of whether reactivation or clearance of protein aggregates is more important for thermotolerance[9]. Remarkably, in Δ*clpB* cells the expression of BAP, ClpB or ClpB and ClpP, but not BAP and ClpP, facilitated survival of severe thermal stress[9]. Importantly, through the assessment of global levels of aggregated protein, BAP and ClpP seemed to be as proficient as ClpB or BAP in clearing protein aggregates after heat shock[9]. Δ*clpB* cells expressing BAP refold luciferase after thermal stress, whereas those expressing BAP and ClpP degrade it, suggesting that refolding aggregated proteins rather than solely eliminating them is essential for cell viability after heat shock[9]. In yeast, it has long been appreciated that reactiva-

tion rather than degradation of aggregated protein is essential for thermotolerance because Hsp104 does not associate with proteolytic systems and substrates are reactivated rather than degraded after heat shock[4]. However, although reactivation is clearly necessary for thermotolerance in *Escherichia coli*, it is unclear whether it is sufficient—that is, there may be additional requirements to eliminate specific toxic aggregates to ensure cell viability. For example, in yeast, under specific circumstances in which Hsp104 and other heat shock protein levels are not elevated in response to elevated temperatures, increased levels of ubiquitin and proteasomal degradation reduce aggregation and enhance cell viability[25].

Much remains to be done in understanding the recalcitrant but universally important subject of protein disaggregation. The brilliant work of Mogk and colleagues[9] provides important new insights, and focuses our future attention on the precise roles played by the middle region of Hsp104/ClpB and its interplay with the Hsp70/DnaK system.

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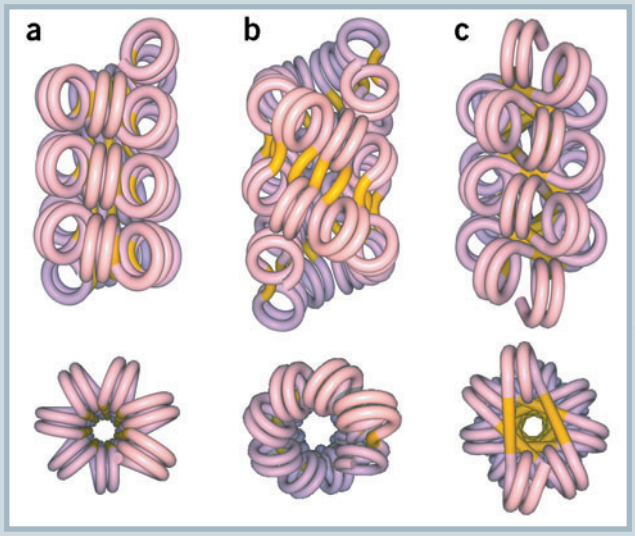
Unraveling chromatin organization

The ordered compaction of DNA is essential to package it within the nucleus (for example, the DNA in a single human cell is more than two meters long). The first order of compaction, the 10-nm fiber, results from the winding of DNA around the core histone octamer. The crystal structure of this particle, the nucleosome, was first solved at a high resolution in 1997. It is composed of two copies each of the four histone subunits 2A, 2B, 3 and 4, which form a flattened disk, wrapped by 146 bp of DNA. Neighboring nucleosomes are separated by a variable length of linker DNA, which can be bound by a linker histone. When viewed in two dimensions, this level of organization has the appearance of beads on a string.

The next order of chromatin compaction has been called the 30-nm fiber. Despite the fact that this structure was first visualized decades ago, the packing organization of nucleosomes within the 30-nm fiber is still debated. Three models have been proposed. In the first model (panel a) neighboring nucleosomes form a continuous helical solenoid, containing about six nucleosomes per turn. The linker DNA (yellow) has the same polarity and is positioned at the central axis. The 'helical ribbon' model (panel b) has the linker DNA joining adjacent particles in neighboring positions of the stacked helices; in this model the linker DNA lies in a groove between the nucleosome cores so that the nucleosomes are not as tightly packed as in the solenoid model. In the 'crossed-linker' model (panel c) the linker DNA is stretched across the central axis, so that neighboring nucleosomes are present on roughly opposite faces of the axis and in different helical stacks. Both the second and third models are known as two-start models, because they involve the linear nucleosome array alternating between two stacked helices.

From previous studies, it was known that the histone H4 tail is required for compaction, and that it interacts with the H2A and H2B subunits of a neighboring nucleosome. To test these packing models, Dorigo *et al.* (*Science* **306**, 1571–1573; 2004) made cysteine substitutions within the H4 tails and H2A/H2B so that higher order chromatin structure could be stabilized by crosslinking. Pairwise combinations of mutant H4 and mutant H2A or H2B were reconstituted *in vitro* as dodecameric nucleosome arrays, and conditions were altered to effect crosslinking and compaction. Only one mutant pair demonstrated a major crosslinked species, and this was employed to examine compaction. When linker DNA was cleaved by a restriction enzyme, the crosslinked species contained 5 or 6 nucleosomes, as would be predicted from the two-start models (panel b and c), but not from the solenoid model (panel a). Next, a 48-mer array of crosslinked particles was visualized by electron microscopy to follow the path of the fiber axis. Many fibers appeared as parallel rows of particles, which dissociated into single columns of particles when the linker DNA was cleaved. This finding supports the two-start models of 30-nm fiber organization, but unfortunately leaves unresolved the question of which two-start model is correct. Higher resolution microscopy will be necessary to resolve these details.



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