

Review Article

AAA+ proteins: one motor, multiple ways to work

JiaBei Lin¹,  James Shorter¹ and  Aaron L. Lucius²

¹Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, U.S.A.; ²Department of Chemistry, University of Alabama at Birmingham, Birmingham, AL, U.S.A.

Correspondence: JiaBei Lin (jiabei@penncmedicine.upenn.edu) or James Shorter (jshorter@penncmedicine.upenn.edu) or Aaron L. Lucius (allucius@uab.edu)

Numerous ATPases associated with diverse cellular activities (AAA+) proteins form hexameric, ring-shaped complexes that function via ATPase-coupled translocation of substrates across the central channel. Cryo-electron microscopy of AAA+ proteins processing substrate has revealed non-symmetric, staircase-like hexameric structures that indicate a sequential clockwise/2-residue step translocation model for these motors. However, for many of the AAA+ proteins that share similar structural features, their translocation properties have not yet been experimentally determined. In the cases where translocation mechanisms have been determined, a two-residue translocation step-size has not been resolved. In this review, we explore Hsp104, ClpB, ClpA and ClpX as examples to review the experimental methods that have been used to examine, in solution, the translocation mechanisms employed by AAA+ motor proteins. We then ask whether AAA+ motors sharing similar structural features can have different translocation mechanisms. Finally, we discuss whether a single AAA+ motor can adopt multiple translocation mechanisms that are responsive to different challenges imposed by the substrate or the environment. We suggest that AAA+ motors adopt more than one translocation mechanism and are tuned to switch to the most energetically efficient mechanism when constraints are applied.

Introduction

ATPases associated with diverse cellular activities (AAA+) play essential roles in cell physiology, such as protein quality control, membrane fusion, DNA replication, repair and recombination, and RNA processing [1–7]. Structurally, they contain a highly conserved ATPase module that harbors α/β and α -helical subdomains in the nucleotide-binding domain (NBD). The Walker A and B motifs of the P-loop on the ATPase module, together with the Sensor 1 motif and Arginine finger, enable nucleotide binding and hydrolysis [4,8]. Most proteins in the AAA+ superfamily utilize the chemical energy provided by ATP binding, hydrolysis, or both to perform mechanical work [4]. However, the motion of the molecular motor that enables mechanical work may vary. In this review, we focus on the mechanisms of AAA+ proteins that are proposed to translocate their polypeptide substrates for the purposes of disaggregation or degradation.

Most AAA+ protein translocases form hexameric ring structures [2,9,10]. From single-particle cryo-electron microscopy (EM) techniques, a number of these proteins form an asymmetric and staircase-like ring arrangement [9,11–20]. In the NBD, a ‘pore-loop’ reaches into the axial channel of the hexameric ring, which contacts substrate directly and is proposed to work like a ‘hand’ that grabs and translocates substrate through the axial channel [9,19]. Generally, two or three structural states were observed for each nucleotide state (ATP, ATP analogs, or ADP), and translocation models were proposed based on these static structural states [11]. Most of the determined structures are from proteins from the classic and HCLR clades of the AAA+ family [4,21], and show that five out of six subunits are engaged with substrate via their pore-loops. The pore-loop contact sites on the substrate are spaced in a two-residue distance. The observation of a ‘seam’ is normally associated with an empty ATP-binding pocket(s) (Apo state) and corresponding pore-loop(s) disengagement of the subunit at the seam. When the ADP and Apo states were only observed at adjacent subunits, a sequential ATP hydrolysis derived hand-over-hand translocation model with a spatiotemporal continuity has been

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proposed [9,11,14,16,17,22]. Specifically, a Sequential Clockwise/2-Residue Step (SC/2R) translocation model has been advanced [9,22]. However, in many example AAA+ molecular motors, these models remain untested in solution. It is also worth noting that there is evidence of plasticity in the mechanism of AAA+ translocases. Indeed, disruption of pore loops or ATP hydrolysis in individual subunits is tolerated to varying degrees without eliminating polypeptide translocation [20,23–25].

The SC/2R translocation model reflects the observed static structural states of a protein translocase. However, the underlying assumption in this model is that the observed structural states lie on the same reaction coordinate. Alternative kinetic paths and patterns of ATP hydrolysis have also been proposed, such as SC/6R and Probabilistic Anti-clockwise Long-Step (PA/LS) models for translocation by ClpX [22,25]. In the PA/LS model, a power stroke in the anti-clockwise direction moves the top subunit to the bottom position yielding a 10-residue step [22]. These diverse structural hypotheses need to be tested in solution for each AAA+ translocase. Dynamic parameters such as rates, rate constants, step-size (amino acids translocated per step), coupling efficiency (ATP hydrolyzed per step) and processivity are outside the temporal resolution of static structural techniques. However, there are a number of biophysical approaches that can determine translocation kinetics and thermodynamics. For instance, the mechanism of translocation used by a mitochondrial inner membrane protease, Yme1, can be evaluated via tracking substrate degradation using chromatography [26–28]. However, AAA+ proteins that might adopt the SC/2R mechanism do not always contain a proteolytic domain, nor do they associate with a proteolytic partner protein. Consequently, since there is no covalent modification of the substrate being translocated, it can be difficult to determine if the motor has passed the substrate through its axial channel.

To test for processivity, methods that detect translocation in the absence of covalent modification are required to examine AAA+ proteins, such as Hsp104, ClpB, p97/VCP, and Vps4. To date, Förster resonance energy transfer (FRET) single-turnover fluorescence/anisotropy/FRET [29–33], hydrogen-deuterium exchange mass-spectrometry [34–36], single-molecule total internal fluorescence spectroscopy [37], and single-molecule optical-trapping techniques [38–41] have been used to interrogate translocation mechanisms. Here, we discuss the translocation mechanism of ClpB and Hsp104 in comparison with other well studied AAA+ protein translocases, such as ClpX and ClpA, which have been scrutinized using a variety of techniques.

Are Hsp104 and ClpB processive translocases?

Hsp104 and its prokaryotic homolog ClpB belong to the Hsp100 Class I family [42]. They are protein disaggregases, which solubilize aggregated structures [18,43]. Hsp104 can even resolve highly stable prions or amyloids as well as neurotoxic preamyloid oligomers [23,43–62]. Hsp104 and ClpB have been hypothesized to operate by the SC/2R mechanism based on cryo-EM structures [9,11,17]. However, large transitions between open lock washer and closed ring structures that could result in a single translocation step of 23 amino acids (AA) are also likely to contribute to activity [9,15,35,36,63–65]. ClpA translocates a polypeptide chain through its axial channel and into ClpP for degradation [66–68]. Polypeptide translocation by ClpA is highly processive, i.e. there is a high probability that ClpA will step forward versus dissociate from the polypeptide substrate [30,32,67]. This observation led to the hypothesis that ClpB and Hsp104, due to the structural similarities to ClpA, would utilize the same mechanism as ClpA to catalyze protein disaggregation [69]. However, neither ClpB nor Hsp104 interact with a chambered protease like ClpP. Consequently, if a polypeptide is processively translocated through their respective axial channels then the polypeptide would leave the reaction without covalent modification, which makes it difficult to determine whether complete translocation has occurred.

In acknowledgement of this experimental difficulty and to test the ‘complete threading’ model, a protein engineering strategy was employed [69,70]. An IGF/L loop on ClpA enables ClpA to interact with the protease ClpP [16]. Thus, the IGF/L loop was engineered into ClpB to enable interaction with ClpP [69]. This modified form of ClpB with an IGF/L loop is termed BAP (ClpB-ClpA-P loop) [69]. If proteolytic degradation of substrates is observed it would suggest that BAP completely threads a polypeptide chain through its axial channel, out the other end, and into the proteolytic chamber of ClpP just like ClpA [69]. Proteolytic degradation of either α -casein or peptide B was observed when mixed with BAP, ClpP and ATP [69]. Degradation of aggregated malate dehydrogenase or aggregated luciferase in the presence of the Hsp70 system (DnaK, DnaJ, and GrpE) was also observed [69].

Interestingly, using the same BAP construct, a later study showed that faster degradation of casein is observed when ATP is omitted from the reaction [30]. This important control was not previously reported [69]. The observation of energy-independent degradation calls into question whether or not the observed

degradation is due to ATPase-driven translocation of a substrate through the axial channel [30]. The observation of ATP-independent degradation does not rule out the possibility that upon addition of ATP there could be a mix of energy-dependent and energy-independent degradation (Figure 1A–C). However, if BAP is a processive translocase that actively translocates a polypeptide through the axial channel and into ClpP for degradation then why would ATP slow this reaction down?

It is important to note that the observation of degradation in that experiment is disappearance of a band at a specific molecular weight on a Coomassie stained SDS–PAGE gel [69]. Thus, this observation only indicates that there is at least one cleavage event that results in a smaller polypeptide but does not, necessarily, indicate complete degradation of a polypeptide substrate as has been shown for ClpA plus ClpP (ClpAP). Thus, the observation of disappearance of a band on a gel does not indicate that BAP translocated the substrate into the proteolytic chamber of ClpP. In contrast, ClpA was shown to fully translocate a polypeptide chain into the cavity of ClpP using stopped flow FRET experiments where a donor fluorophore is placed in the ClpP cavity, and an acceptor fluorophore is placed on the substrate being translocated [30,68]. However, when this strategy was applied to BAP–ClpP no FRET signal was observed under conditions where degradation monitored by a loss of a band on a gel is observed [30]. These observations suggest that BAP is not sufficiently processive to fully pass a polypeptide chain through its axial channel and into ClpP [30]. However, some degradation occurs as indicated by disappearance of the band on the gel.

Single-turnover fluorescence/Anisotropy/FRET experiments to determine the path of translocation

Single turnover transient state kinetic techniques have been developed to examine polypeptide translocation catalyzed by ClpA [67], ClpB [30], Hsp104, [29] and N-ethylmaleimide-sensitive Factor (NSF) [71]. The protein translocase (e.g. ClpA) is pre-bound to its fluorescently-labeled unstructured substrate in the presence of a non-hydrolyzable or slowly hydrolyzable nucleotide analog, typically ATP γ S, in one syringe (Figure 2A). No active translocation should occur as there is no rapidly hydrolyzable ATP present and thus no energy provided by ATP hydrolysis. In the other syringe, there is a large excess (relative to the non-hydrolyzable nucleotide) of ATP and unlabeled polypeptide substrate (relative to the fluorescently-labeled substrate; Figure 2A). The

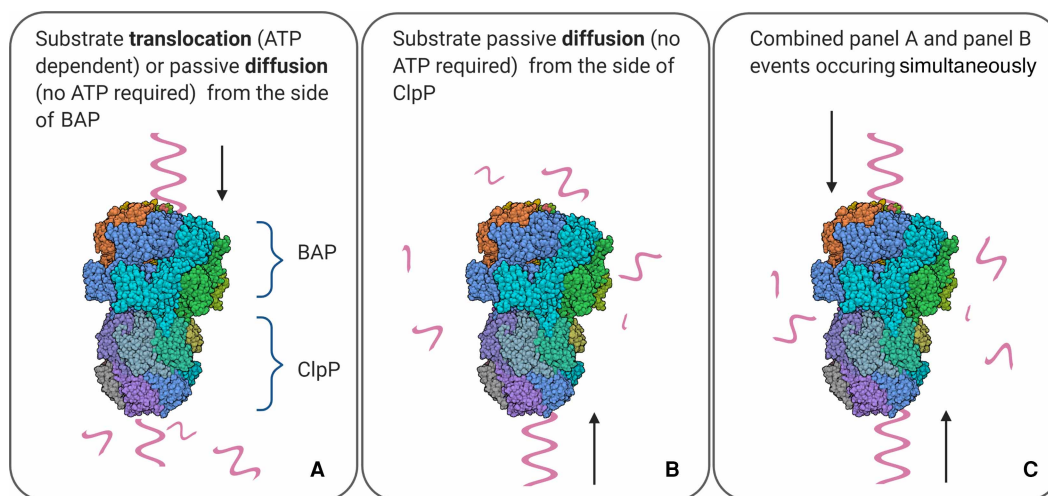


Figure 1. Interpretation of the degradation mechanism of BAP–ClpP system.

(A) ClpAP structure (6W20) was used to mimic the BAP–ClpP complex. The ATP-dependent, processive translocation of substrate through the BAP central pore into the ClpP chamber is depicted. Alternatively, passive diffusion of the polypeptide into ClpP might occur from the BAP side in an ATP-independent manner. Peptide substrate is represented by magenta lines, the motor protein, BAP, and the protease, ClpP, are indicated in the scheme. (B) When no ATP is required for substrate degradation of BAP–ClpP, polypeptide degradation might be explained by passive diffusion of polypeptide into the ClpP chamber from the BAP side (A) or the ClpP side (B). (C) ATP-dependent translocation and ATP-independent passive diffusion from the BAP or ClpP side could all occur at the same time and result in substrate degradation.

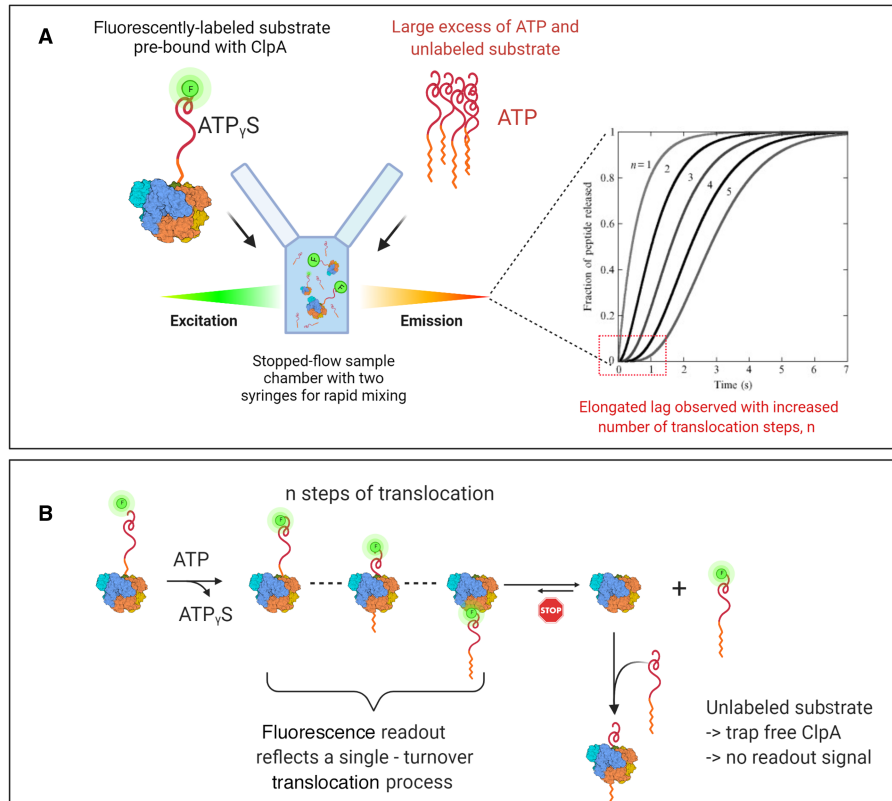


Figure 2. A scheme of single-turnover experiments to measure protein translocation.

(A) Schematic cartoon of stopped flow fluorescence measurements. ClpA hexamer pre-bound with fluorescently-labeled substrate in the presence of ATP γ S (left side syringe) was rapidly mixed with ATP and unlabeled substrate (in large excess in right side syringe). Fluorescence measurements were taken as a function of time. To test the processivity of the motor protein, multiple lengths of substrates should be tested. The observed length of the lag phase of the fluorescence time course should appear to be positively correlated to the length of the substrate for processive translocation. (B) The reaction scheme of the measurements taken place in panel A. A preformed fluorescently-labeled substrate -ClpA -ATP γ S complex was subjected to a large excess of ATP. ATP replaces the prebound ATP γ S and initiates translocation. It takes n -steps ($n \geq 1$) for ClpA to translocate the substrate completely. Once dissociated, the fluorescently-labeled substrate cannot rebind to ClpA due to the presence of large excess of unlabeled substrate. Thus, the detected signal reports a single-turnover event of substrate translocation.

contents of the two syringes are rapidly mixed within 2 ms. ClpA binding to the substrate results in a change in fluorescence (or fluorescence anisotropy [72]), which is monitored as a function of time. As $[ATP] \gg [ATP\gamma S]$, ATP will replace ATP γ S and hydrolyze to initiate the translocation reaction (Figure 2B). The motor will proceed for n steps before reaching the end of the polypeptide chain and dissociating or it can dissociate at any intermediate step before reaching the end. The dissociated motor cannot re-bind to the fluorescently-labeled substrate as it will be trapped by the unlabeled, 'dark' substrate as $[dark\ substrate] \gg [labeled\ substrate]$, which assures that the time courses reflect only a single-turnover of polypeptide translocation.

If we define processivity as a probability that an enzyme will step forward versus dissociate then a processivity, $P = 1$, means that the enzyme will always reach the end of the lattice regardless of its length. Thus, to test for processive translocation the single turnover experimental strategy described in Figure 2 is performed as a function of polypeptide substrate length. The prediction is that for processive translocases the time courses will depend on substrate length. If the lattice length is increased, the enzyme will take more steps to completely translocate the increased lattice length. Therefore, the number of observed steps, n , will depend on the lattice length, L , based on the distance traveled per step, m (step size), as $n = L/m$. A lag in signal that increases with lattice length is often observed (Figure 2A). However, the fundamental prediction is that if the enzyme is

processively translocating a substrate then it will be taking increasing number of steps with increasing substrate length. Consequently, the time courses will indicate a length dependence for a processive translocase. In contrast, if a motor exhibits sufficiently low processivity, $P \ll 1$, then no length dependence will be observed.

ClpA processively translocates an unstructured polypeptide chain through its axial channel and into ClpP [67]. The fluorescence stopped flow method described above was developed to examine unstructured polypeptide translocation catalyzed by ClpA in the absence of ClpP. This translocation scenario would be analogous to translocation catalyzed by ClpB or Hsp104 that do not interact with a protease. Using the method in Figure 2A, time courses were reported that showed a clear length dependence [67]. From an analysis of these time courses, a molecular mechanism for translocation catalyzed by ClpA was reported that included the first determination of a kinetic step-size for a AAA+ molecular motor to be ~ 14 AA step^{-1} independent of [ATP] [67]. Interestingly, the same stopped-flow experimental strategy, when applied to ClpAP, revealed a kinetic step-size of ~ 5 AA step^{-1} , revealing that ClpP allosterically influences the mechanism of ClpA-catalyzed polypeptide translocation, consistent with mechanistic plasticity [32]. It is important to note that the kinetic step-size represents the average number of amino acids translocated between two rate-limiting steps and further experiments are required to determine if it represents mechanical movement. However, a later study using optical tweezers and folded substrates showed that ClpAP translocated with a mechanical step size between 4–8 amino acids lending support to the notion that the kinetic step-size is likely reporting on mechanical movement [73].

The transient state kinetic analysis on ClpA and ClpAP revealed the stopped flow technique to be a viable strategy for examining polypeptide translocation in the absence of a proteolytic partner [32,67]. This technique was subsequently used to examine translocation catalyzed by wild-type ClpB [30]. Stopped-flow experiments were performed with ClpB on multiple substrate lengths ranging from 30 to 102 amino acids [30]. Interestingly, no length dependent time courses were ever observed [30]. It was concluded that ClpB proceeded through 1–2 rate-limiting steps before dissociation from the polypeptide substrate, regardless of the length [30]. This finding was confirmed using fluorescence, fluorescence anisotropy, and FRET as the observed signals, all of which are sensitive to different aspects of movement of the motor [30].

Figure 2A presents fluorescence as the detection signal. However, if there is no change in the total fluorescence signal upon motor dissociation, then anisotropy can be used. If the size of substrate is too large to result in a significant anisotropy change upon dissociation, FRET is another choice for measuring the translocation. In this scenario, the motor should also be labeled with a fluorophore that is a FRET pair of the label on the substrate.

Durie et al. tested polypeptide translocation catalyzed by Hsp104 using the fluorescence stopped flow design described above [29]. In this study, substrates with various lengths of 30, 40, 50, 102, and 127 amino acids were used to determine the number of translocation steps catalyzed by Hsp104 [29]. In this work, similar to ClpB, no length dependence was observed. Thus, the authors proposed that Hsp104 can operate as a non-processive translocase [29]. Given that no more than two translocation steps were observed for tested substrates of various lengths, a ‘toggle and release’ model was proposed for the mechanism of Hsp104-mediated protein disaggregation. However, it is important to note that in both these studies [29,30], one or two slow steps followed by very fast translocation would be difficult to detect on the short substrates used. Thus, it will be of great interest to assess Hsp104 and ClpB processivity on longer polypeptide substrates. Further, all substrates examined were unstructured so an important next step is to challenge the motor with folded regions.

Single-molecule optical-trap experiments to determine the path of translocation

Single-molecule optical trap experiments enable us to study the force generation, processivity, rate and step size of single enzymes as a function of nucleotide concentration and applied force. Compared to ensemble experiments, this approach allows the detection of the heterogeneity of translocation events of each individual enzyme rather than measuring the average events of all enzymes in solution. The first measurements of Hsp100-driven protein translocation using this technique were on a Class II Hsp100 enzyme, ClpX, with its proteolytic partner, ClpP [38,74]. In one study, ClpX plus ClpP (ClpXP) and a multidomain substrate (filamin A domains 1–8) with an SsrA-tag were tethered between two polystyrene beads trapped by optical lasers [38]. ClpX and ClpA recognize the SsrA-tag (even on internal loops) and initiate translocation [75]. Different from ClpX and ClpA, ClpB does not recognize any specific linear sequence akin to the SsrA-tag, and may prefer to bind to unstructured polypeptides with some charge [76,77]. Interestingly, BAP-ClpP was proposed to partially

translocate a looped polypeptide segment of a CFP-luciferase-YFP sandwich construct (Figure 3A), and its degradation products were evaluated using SDS-PAGE gel and LC-MS/MS analysis [78]. The denatured luciferase segment was targeted and degraded incompletely upon BAP-ClpP treatment, whereas the stable CFP and YFP flank remained properly folded and were not degraded [78]. By contrast, a related Hsp100 protein, ClpC, which engages ClpP could unfold the CFP and YFP portions and promote their degradation by ClpP [78]. Notably, ClpB hexamers are dynamic assemblies, with a dissociation rate constant as fast as 0.85 min^{-1} [78]. Given that the stability of BAP hexamer in the presence of ClpP was not investigated, the possibility of free BAP assembling around a single peptide strand of the CFP-luciferase-YFP and bringing it to the ClpP chamber for cleavage remains to be tested (Figure 3B) [78]. The detection of a large fraction of luciferase (157 AA) – YFP fragments after 60 min of the cleavage reactions of CFP-Luciferase-YFP and Luciferase-YFP indicates a partial translocation of the substrates [78]. Thus, ClpB might specifically target unfolded portions of protein aggregates for translocation and then release upon sensing a stably folded domain [78]. ClpB may alternate between processive translocation on unfolded segments and then release, such that overall translocation by ClpB is non-processive. That is, $P = 1$ on unfolded segments, but $P = 0$ for folded segments. Thus, processive translocation by ClpB may be context specific and sensitive to the properties of the substrate. However, it should be noted that the amount of ATP-independent degradation induced by BAP-ClpP in these experiments was not accounted for in the interpretation of the observations.

A distinct, partial translocation mechanism involving unfoldase activity enables Hsp104 to dissolve Sup35 prions [43,58]. Here, Hsp104 engages the assembled Sup35 prion between Sup35 residues 96–151, at the C-terminal end of the N domain and N-terminal end of the M domain of Sup35 [43,58]. After this initial engagement, Hsp104 then translocates toward the N-terminus of Sup35, thereby unfolding the cross- β core of the prion without disrupting the folded C-terminal GTPase domain of Sup35 (residues 254–685) [43,58]. In this case, Hsp104 may processively translocate only the prion domain of Sup35 and not the C-terminal GTPase domain providing an example of processive, but still partial translocation [43,58]. In this way, soluble, functional Sup35 is rapidly released from the prion state [43,58]. Hsp104 has also been proposed to utilize a partial translocation mechanism to efficiently disperse Pab1 condensates [79]. These examples illustrate that translocation mechanism may be context specific and depend on the nature of the substrate.

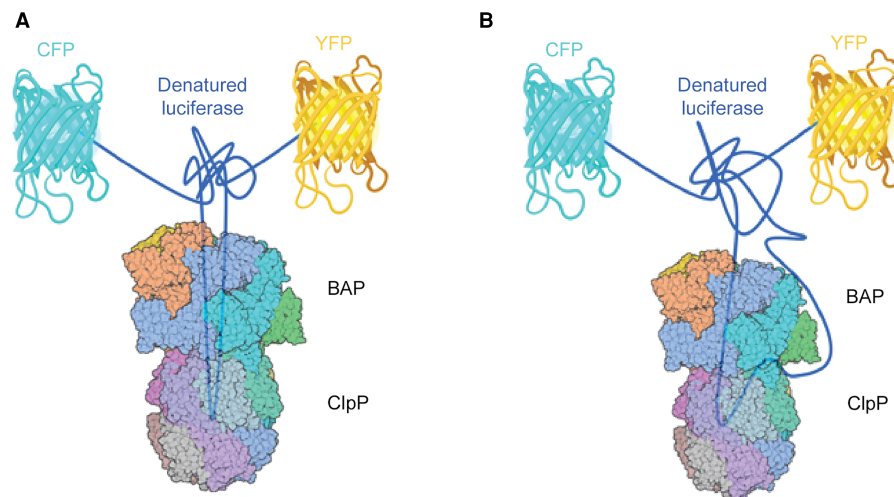


Figure 3. Partial threading of a looped peptide and a single-strand peptide through the central pole of BAP.

(A) BAP hexamer translocates a banded protein loop into its central pore and into ClpP. CFP and YFP are shown in cyan and yellow. CFP and YFP are properly folded and thus not recognized by BAP as a substrate. To engage the denatured region in the middle, BAP may bind to an unfolded protein loop. (B) BAP hexamer assembles around a single polypeptide chain. ClpB hexamers exhibit rapid dissociation and reassociation in the presence of ATP [94]. Therefore, BAP may assemble around a single peptide strand, and engage in the central pore (the left luciferase strand), whereas the remainder of denatured luciferase is outside the central pore. This preformed BAP-luciferase complex may then deliver polypeptide into ClpP.

ClpX, ClpA and ClpB share similar structural features [16,17,22,80,81], which suggest that they may also employ the SC/2R translocation mechanism. These proteins differ in hexamer stability, partner-protein selection, and substrate recognition and translocation [30,38,39,67,68,75,77,82,83]. Both ClpA and ClpX can recognize the SsrA-tag to initiate translocation through their axial channels (Figure 4A) [38,75]. Thus, single-molecule trap experiments were performed using similar strategies for ClpX (Figure 4A). A single-chain ClpX complex was tethered to one polystyrene bead and the peptide substrate linked to an SsrA-tag to the other bead. The motor protein can unfold and translocate properly structured protein (Figure 4A). ClpXP translocates substrate in step sizes ranging from 5–8 AA to 10–13 AA [38]. It was suggested that ClpXP uses a low gear to pull against a relatively high load and operates by a power-stroke translocation mechanism [38]. It is important to note that single-molecule experiments and single-turnover experiments cannot exclude the possibility of rapid small substeps (e.g. 2 AA proposed in the SC/2R model) that blur into a single step followed by a slow step.

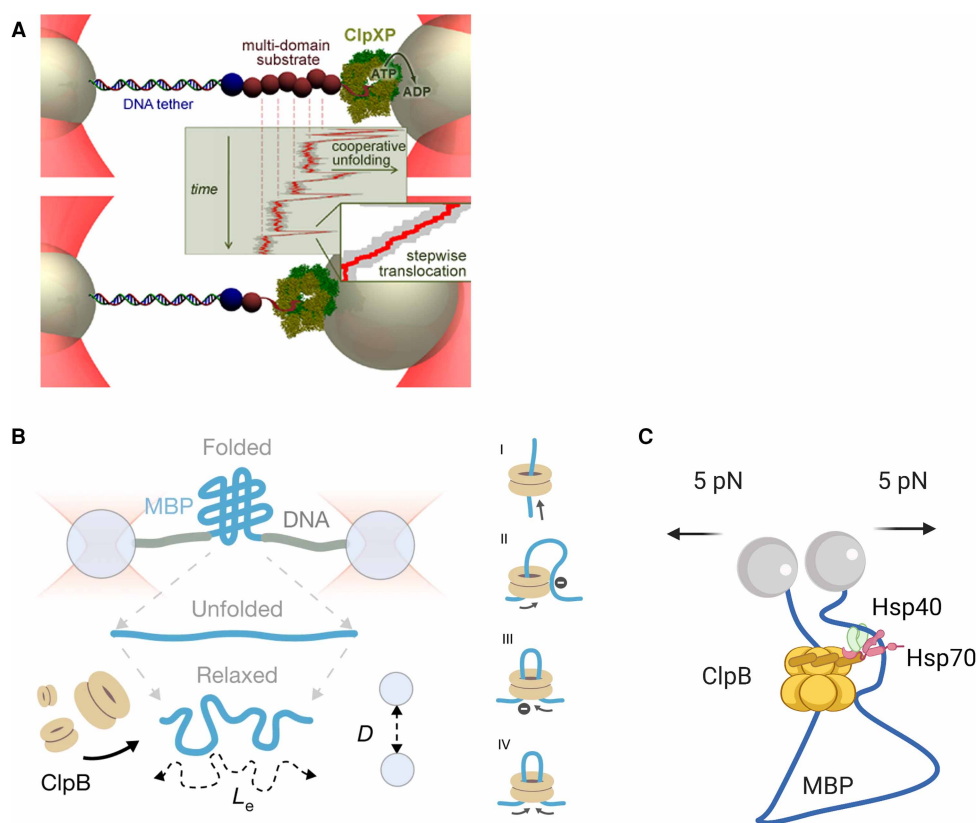


Figure 4. Distinct strategies to study substrate translocation by ClpXP and ClpB.

(A) ClpXP translocates a single-strand protein substrate [38]. A multi-domain substrate was covalently attached to the bead through a DNA linker. ClpXP complex was covalently attached to another bead, where both beads were trapped by optical tweezers. The distance change of two beads as a function of time can be measured, which is directly connected to ClpXP translocation and substrate unfolding [38]. Adapted from [38] with permission. (B) ClpB translocates a double-strand protein loop [39]. A MBP protein was tethered in between two beads that are trapped by optical tweezers. More than one ClpB hexamer was allowed to engage with unfolded MBP. Four possible modes of MBP-ClpB engagement: I, a single-strand peptide was engaged in the central pore of ClpB and can be translocated. II, a single-strand peptide was engaged in the central pore of ClpB, and another side of the substrate binds to the exterior of ClpB. Only the central pore of ClpB allows relative movement between ClpB and substrate. III, a peptide loop engaged in the central pore and only one strand of the loop can be translocated. IV, a peptide loop engaged in the central pore and both strands of the loop can be translocated. Adapted from [39] with permission. (C) A cartoon shows that ClpB translocates a single-strand substrate with an M-domain engaged, resulting in the distance change of the optical beads. The M-domain of ClpB is known to be flexible and interacts with Hsp70 [17,88,95,96]. This feature allows the M-domain to assist the substrate translocation in the central pore and results in a distance change of the optical beads.

Single molecule optical tweezer experiments have suggested that ClpB hyperactive variants, ClpB^{Y503D} and ClpB^{K476C}, and wild-type ClpB in the presence of DnaK, DnaJ, and GrpE translocate a polypeptide substrate that was constrained at both ends [39]. In this design, the polypeptide was tethered to DNA handles that were both attached to polystyrene beads, which were optically trapped (Figure 4B) [39]. Thus, ClpB would not encounter a free N- or C-terminus [39]. A rapid reduction in the distance between the two beads occurred upon addition of 2 μ M ClpB plus ATP [39]. Two rates were reported for ClpB^{Y503D}, ~250 and 450 amino acids per second [39]. It is important to note that this experiment is single molecule with respect to the polypeptide chain and not ClpB [39]. Since ClpB is in large excess it is unlikely that only one hexamer is binding and doing the work. It seems more likely that ClpB is condensing on the substrate since the extremely high rates are independent of the forces applied, which exceeded 50 pN [39]. In analogous fluorescence experiments, it was concluded that a single ClpB hexamer was recruited based on the observation of one fluorophore [39]. However, the labeling efficiency of the monomers is not reported [39], and it is unlikely to be 100%. In fact, the observation of only one fluorophore on one hexamer reveals an upper limit on the labeling efficiency of 1/6 or ~16%, which indicates that at 2 μ M ClpB there would be 1.7 μ M unlabeled molecules that are capable of condensing on the substrate. A substantial excess relative to a single polypeptide chain.

Four types of polypeptide extrusion models were considered (Figure 4B, models I-IV) [39]. It was concluded that ClpB can translocate both arms of the loop simultaneously (model IV), but is capable of single-arm translocation when obstacles are encountered (model III) [39]. Consistent with this possibility, other AAA+ proteins, such as Vps4 [84], p97/VCP [85] and ClpX [86,87], can translocate circular or knotted polypeptides, indicating that AAA+ ATPases may translocate diverse substrates including loops, knots, hairpins, and crosslinked polypeptides. However, it remains possible that two-arm translocation might proceed via a non-canonical mechanism on the exterior of the ClpB hexamer, perhaps involving the M domain (Figure 4C) [88]. The fact that ClpB stays on the MBP substrate when the MBP substrate is fully stretched repetitively [39], makes the model presented in Figure 4C a possible alternative mechanism for the substrate extrusion. Fully stretching MBP would be expected to eject ClpB from substrate according to model III and IV (Figure 4B). It will be interesting to establish whether ClpB can also translocate circular peptides as Vps4 has been reported to do [84]. It will also be important to determine the binding affinities of the double-stranded and single-stranded polypeptides to ClpB.

Avellaneda et al. [39] present strong evidence that ClpB is a processive and powerful translocase that can pull against forces as high as 50 pN at a speed of ~240 AA per second of a looped polypeptide. The energy required to do this work is $\sim 7.2 \times 10^{-18}$ J per second for one ClpB hexamer molecule (Work = Force*Distance) [39]. Thus, at least ~72 ATP molecules (one ATP contributes ~60 kJ/mol [89–91]) need to be hydrolyzed by one ClpB hexamer per second at the time of translocation assuming all the energy comes from ATP hydrolysis and binding energy makes minimal contribution. However, the highest ATP hydrolysis reported for ClpB-Y503D is 6 ATP per second per ClpB [39]. Of course, the reported ATPase activity in the presence of a large excess of substrate could be an underestimate of the working ClpB hexamer. However, this large discrepancy leads to the question: is more than one ClpB hexamer driving the observed peptide extrusion? It is worth noting that this measurement is of extrusion of a looped peptide of a single MBP molecule, but not likely the work of a single ClpB hexameric motor. We previously established that more than one hexamer would bind to an unfolded polypeptide chain once the length exceeded 127 amino acids [77]. Thus, the mechanically stretched and unfolded MBP would accommodate multiple hexamers at such a large excess of ClpB.

The observed polypeptide extrusion steps reported for ClpB are much larger than two AA per step, even in the presence of a mixture of ATP and ATP γ S in an effort of slowing down the rate of ATP hydrolysis [39]. The minimum step size observed can be limited by the time resolution of optical-trap setup. For the reported rate, a 2 AA step would occur in ~8 ms, whereas the detected 14 AA constant steps occurred every ~40–60 ms after taking time-average of the measurements [39]. Notably, single-molecule FRET work has suggested that ClpB adopts a Brownian ratchet translocation mechanism and ultrafast pore-loop movements that occur more rapidly than ATP hydrolysis may help drive polypeptide translocation [92]. In this work, the relative motion of substrate-binding pore loops with respect to their NBDs was measured using single-molecule FRET [92]. The pore loops exhibit large-amplitude fluctuations on the microsecond timeframe and change their conformation upon substrate engagement and ATP hydrolysis, indicating that these motions might drive substrate translocation [92]. The ultrafast local Brownian ratcheting of pore-loops in combination of hand-over-hand motion ClpB may explain the large discrepancy between the cryo-EM translocation model with a 2-AA step size and the 14-AA translocation step size determined by single-molecule optical-trap [9,17,39,65]. Nonetheless, an experimental determination of hand-over-hand motion of ClpB is still missing.

Closing remarks

In our view, the direct measurements of Hsp104 or ClpB translocation remain insufficient to support the hypothesis that Hsp104 or ClpB translocates substrate using an invariably identical mechanism to ClpA or ClpX. Instead, when parallel experiments were performed side by side, using ClpAP and BAP-ClpP, translocation was only observed for ClpA but not BAP [30]. Moreover, Hsp104 adopts distinct disaggregation mechanisms in a substrate-dependent manner [23,45,93]. Cooperativity between subunits is not necessarily required to disaggregate misfolded amorphous aggregates, but it is essential to disaggregate substrates with more stable cross- β cores, such as Ure2 prions or α -synuclein amyloid [23,93]. This operational plasticity seems to rule out an SC/2R as an exclusive mechanism that must always be utilized to drive disaggregation, although it could be used in specific circumstances. We suggest that Hsp104 and other AAA+ proteins exhibit plasticity in response to different substrates and may only work as processive translocases when needed.

Perspectives

- How hexameric, ring-shaped AAA+ proteins translocate polypeptide substrates remains incompletely understood.
- Cryo-EM and single-molecule studies suggest distinct mechanisms of polypeptide translocation.
- We suggest that AAA+ motors may adopt more than one translocation mechanism and are tuned to switch to the most energetically efficient mechanism when constraints are applied.

Competing Interests

J.B.L. and A.L.L. have nothing to declare. J.S. is a consultant for Dewpoint Therapeutics, Vivid Sciences, Korro Bio, ADRx, and Neumora.

Author Contributions

J.B.L., J.S., and A.L.L. wrote the manuscript.

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Abbreviations

AA, amino acids; EM, electron microscopy; FRET, Förster resonance energy transfer; NBD, nucleotide-binding domain.

References

- 1 Ogura, T. and Wilkinson, A.J. (2001) AAA+ superfamily ATPases: common structure—diverse function. *Genes Cells* **6**, 575–597 <https://doi.org/10.1046/j.1365-2443.2001.00447.x>
- 2 Snider, J., Thibault, G. and Houry, W.A. (2008) The AAA+ superfamily of functionally diverse proteins. *Genome Biol.* **9**, 216 <https://doi.org/10.1186/gb-2008-9-4-216>
- 3 Shorter, J. and Houry, W.A. (2018) Editorial: the role of AAA+ proteins in protein repair and degradation. *Front. Mol. Biosci.* **5**, 85 <https://doi.org/10.3389/fmolb.2018.00085>
- 4 Seraphim, T.V. and Houry, W.A. (2020) AAA+ proteins. *Curr. Biol.* **30**, R251–R257 <https://doi.org/10.1016/j.cub.2020.01.044>
- 5 Khan, Y.A., White, K.I. and Brunger, A.T. (2021) The AAA+ superfamily: a review of the structural and mechanistic principles of these molecular machines. *Crit. Rev. Biochem. Mol. Biol.*, 1–32 <https://doi.org/10.1080/10409238.2021.1979460>
- 6 Cupo, R.R. and Shorter, J. (2020) Skd3 (human ClpB) is a potent mitochondrial protein disaggregase that is inactivated by 3-methylglutaconic aciduria-linked mutations. *eLife* **9**, e55279 <https://doi.org/10.7554/eLife.55279>

- 7 Warren, J.T., Cupo, R.R., Wattanasirakul, P., Spencer, D.H., Locke, A.E., Makaryan, V. et al. (2022) Heterozygous variants of CLPB are a cause of severe congenital neutropenia. *Blood* **139**, 779–791 <https://doi.org/10.1182/blood.2021010762>
- 8 Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945–951 <https://doi.org/10.1002/j.1460-2075.1982.tb01276.x>
- 9 Gates, S.N., Yokom, A.L., Lin, J., Jackrel, M.E., Rizo, A.N., Kendsersky, N.M. et al. (2017) Ratchet-like polypeptide translocation mechanism of the AAA+ disaggregase Hsp104. *Science* **357**, 273–279 <https://doi.org/10.1126/science.aan1052>
- 10 Tang, W.K., Borgnia, M.J., Hsu, A.L., Esser, L., Fox, T., de Val, N. et al. (2020) Structures of AAA protein translocase Bcs1 suggest translocation mechanism of a folded protein. *Nat. Struct. Mol. Biol.* **27**, 202–209 <https://doi.org/10.1038/s41594-020-0373-0>
- 11 Gates, S.N. and Martin, A. (2020) Stairway to translocation: AAA+ motor structures reveal the mechanisms of ATP-dependent substrate translocation. *Protein Sci.* **29**, 407–419 <https://doi.org/10.1002/pro.3743>
- 12 Banerjee, S., Bartesaghi, A., Merk, A., Rao, P., Bulfer, S.L., Yan, Y. et al. (2016) 2.3 Å resolution cryo-EM structure of human p97 and mechanism of allosteric inhibition. *Science* **351**, 871–875 <https://doi.org/10.1126/science.aad7974>
- 13 Twomey, E.C., Ji, Z., Wales, T.E., Bodnar, N.O., Ficarro, S.B., Marto, J.A. et al. (2019) Substrate processing by the Cdc48 ATPase complex is initiated by ubiquitin unfolding. *Science* **365**, eaax1033 <https://doi.org/10.1126/science.aax1033>
- 14 Puchades, C., Rampello, A.J., Shin, M., Giuliano, C.J., Wiseman, R.L., Glynn, S.E. et al. (2017) Structure of the mitochondrial inner membrane AAA+ protease YME1 gives insight into substrate processing. *Science* **358**, eaao0464 <https://doi.org/10.1126/science.aao0464>
- 15 Yokom, A.L., Gates, S.N., Jackrel, M.E., Mack, K.L., Su, M., Shorter, J. et al. (2016) Spiral architecture of the Hsp104 disaggregase reveals the basis for polypeptide translocation. *Nat. Struct. Mol. Biol.* **23**, 830–837 <https://doi.org/10.1038/nsmb.3277>
- 16 Lopez, K.E., Rizo, A.N., Tse, E., Lin, J., Scull, N.W., Thwin, A.C. et al. (2020) Conformational plasticity of the ClpAP AAA+ protease couples protein unfolding and proteolysis. *Nat. Struct. Mol. Biol.* **27**, 406–416 <https://doi.org/10.1038/s41594-020-0409-5>
- 17 Rizo, A.N., Lin, J., Gates, S.N., Tse, E., Bart, S.M., Castellano, L.M. et al. (2019) Structural basis for substrate gripping and translocation by the ClpB AAA+ disaggregase. *Nat. Commun.* **10**, 2393 <https://doi.org/10.1038/s41467-019-10150-y>
- 18 Shorter, J. and Southworth, D.R. (2019) Spiraling in control: structures and mechanisms of the Hsp104 disaggregase. *Cold Spring Harb. Perspect. Biol.* **11**, a034033 <https://doi.org/10.1101/cshperspect.a034033>
- 19 Kim, Y.C., Snoberger, A., Schupp, J. and Smith, D.M. (2015) ATP binding to neighbouring subunits and intersubunit allosteric coupling underlie proteasomal ATPase function. *Nat. Commun.* **6**, 8520 <https://doi.org/10.1038/ncomms9520>
- 20 Cupo, R.R., Rizo, A.N., Braun, G.A., Tse, E., Chuang, E., Southworth, D.R. et al. (2022) Unique structural features govern the activity of a human mitochondrial AAA+ disaggregase, Skd3. *bioRxiv* <https://doi.org/10.1101/2022.02.17.480866>
- 21 Erzberger, J.P. and Berger, J.M. (2006) Evolutionary relationships and structural mechanisms of AAA+ proteins. *Annu. Rev. Biophys. Biomol. Struct.* **35**, 93–114 <https://doi.org/10.1146/annurev.biophys.35.040405.101933>
- 22 Fei, X., Bell, T.A., Jenni, S., Stinson, B.M., Baker, T.A., Harrison, S.C. et al. (2020) Structures of the ATP-fueled ClpXP proteolytic machine bound to protein substrate. *eLife* **9**, e52774 <https://doi.org/10.7554/eLife.52774>
- 23 DeSantis, M.E., Leung, E.H., Sweeny, E.A., Jackrel, M.E., Cushman-Nick, M., Neuhaus-Follini, A. et al. (2012) Operational plasticity enables Hsp104 to disaggregate diverse amyloid and nonamyloid clients. *Cell* **151**, 778–793 <https://doi.org/10.1016/j.cell.2012.09.038>
- 24 Martin, A., Baker, T.A. and Sauer, R.T. (2005) Rebuilt AAA+motors reveal operating principles for ATP-fuelled machines. *Nature* **437**, 1115–1120 <https://doi.org/10.1038/nature04031>
- 25 Sauer, R.T., Fei, X., Bell, T.A. and Baker, T.A. (2021) Structure and function of ClpXP, a AAA+ proteolytic machine powered by probabilistic ATP hydrolysis. *Crit. Rev. Biochem. Mol. Biol.*, 1–17 <https://doi.org/10.1080/10409238.2021.1979461>
- 26 Leonhard, K., Stiegler, A., Neupert, W. and Langer, T. (1999) Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature* **398**, 348–351 <https://doi.org/10.1038/18704>
- 27 Rainey, R.N., Glavin, J.D., Chen, H.W., French, S.W., Teitell, M.A. and Koehler, C.M. (2006) A new function in translocation for the mitochondrial i-AAA protease Yme1: import of polynucleotide phosphorylase into the intermembrane space. *Mol. Cell. Biol.* **26**, 8488–8497 <https://doi.org/10.1128/MCB.01006-06>
- 28 Shi, H., Rampello, A.J. and Glynn, S.E. (2016) Engineered AAA+ proteases reveal principles of proteolysis at the mitochondrial inner membrane. *Nat. Commun.* **7**, 13301 <https://doi.org/10.1038/ncomms13301>
- 29 Durie, C.L., Lin, J., Scull, N.W., Mack, K.L., Jackrel, M.E., Sweeny, E.A. et al. (2019) Hsp104 and potentiated variants can operate as distinct nonprocessive translocases. *Biophys. J.* **116**, 1856–1872 <https://doi.org/10.1016/j.bpj.2019.03.035>
- 30 Li, T., Weaver, C.L., Lin, J., Duran, E.C., Miller, J.M. and Lucius, A.L. (2015) *Escherichia coli* ClpB is a non-processive polypeptide translocase. *Biochem. J.* **470**, 39–52 <https://doi.org/10.1042/BJ20141457>
- 31 Lucius, A.L., Maluf, N.K., Fischer, C.J. and Lohman, T.M. (2003) General methods for analysis of sequential “n-step” kinetic mechanisms: application to single turnover kinetics of helicase-catalyzed DNA unwinding. *Biophys. J.* **85**, 2224–2239 [https://doi.org/10.1016/S0006-3495\(03\)74648-7](https://doi.org/10.1016/S0006-3495(03)74648-7)
- 32 Miller, J.M., Lin, J., Li, T. and Lucius, A.L. (2013) *E. coli* ClpA catalyzed polypeptide translocation is allosterically controlled by the protease ClpP. *J. Mol. Biol.* **425**, 2795–2812 <https://doi.org/10.1016/j.jmb.2013.04.019>
- 33 Bjornson, K.P., Amaratunga, M., Moore, K.J. and Lohman, T.M. (1994) Single-turnover kinetics of helicase-catalyzed DNA unwinding monitored continuously by fluorescence energy transfer. *Biochemistry* **33**, 14306–14316 <https://doi.org/10.1021/bi00251a044>
- 34 Yang, B., Stjepanovic, G., Shen, Q., Martin, A. and Hurley, J.H. (2015) Vps4 disassembles an ESCRT-III filament by global unfolding and processive translocation. *Nat. Struct. Mol. Biol.* **22**, 492–498 <https://doi.org/10.1038/nsmb.3015>
- 35 Ye, X., Lin, J., Mayne, L., Shorter, J. and Englander, S.W. (2019) Hydrogen exchange reveals Hsp104 architecture, structural dynamics, and energetics in physiological solution. *Proc. Natl Acad. Sci. U.S.A.* **116**, 7333–7342 <https://doi.org/10.1073/pnas.1816184116>
- 36 Ye, X., Lin, J., Mayne, L., Shorter, J. and Englander, S.W. (2020) Structural and kinetic basis for the regulation and potentiation of Hsp104 function. *Proc. Natl Acad. Sci. U.S.A.* **117**, 9384–9392 <https://doi.org/10.1073/pnas.1921968117>
- 37 Catipovic, M.A., Bauer, B.W., Loparo, J.J. and Rapoport, T.A. (2019) Protein translocation by the SecA ATPase occurs by a power-stroke mechanism. *EMBO J.* **38**, e101140 <https://doi.org/10.15252/emj.2018101140>

- 38 Aubin-Tam, M.E., Olivares, A.O., Sauer, R.T., Baker, T.A. and Lang, M.J. (2011) Single-molecule protein unfolding and translocation by an ATP-fueled proteolytic machine. *Cell* **145**, 257–267 <https://doi.org/10.1016/j.cell.2011.03.036>
- 39 Avellaneda, M.J., Franke, K.B., Sunderlikova, V., Bukau, B., Mogk, A. and Tans, S.J. (2020) Processive extrusion of polypeptide loops by a Hsp100 disaggregase. *Nature* **578**, 317–320 <https://doi.org/10.1038/s41586-020-1964-y>
- 40 Chen, C., Stevens, B., Kaur, J., Cabral, D., Liu, H., Wang, Y. et al. (2011) Single-molecule fluorescence measurements of ribosomal translocation dynamics. *Mol. Cell* **42**, 367–377 <https://doi.org/10.1016/j.molcel.2011.03.024>
- 41 Sun, Y., Sato, O., Ruhnnow, F., Arsenaault, M.E., Ikebe, M. and Goldman, Y.E. (2010) Single-molecule stepping and structural dynamics of myosin X. *Nat. Struct. Mol. Biol.* **17**, 485–491 <https://doi.org/10.1038/nsmb.1785>
- 42 Schirmer, E.C., Glover, J.R., Singer, M.A. and Lindquist, S. (1996) HSP100/Cip proteins: a common mechanism explains diverse functions. *Trends Biochem. Sci.* **21**, 289–296 [https://doi.org/10.1016/S0968-0004\(96\)10038-4](https://doi.org/10.1016/S0968-0004(96)10038-4)
- 43 Sweeny, E.A. and Shorter, J. (2016) Mechanistic and structural insights into the prion-disaggregase activity of Hsp104. *J. Mol. Biol.* **428**, 1870–1885 <https://doi.org/10.1016/j.jmb.2015.11.016>
- 44 Castellano, L.M., Bart, S.M., Holmes, V.M., Weissman, D. and Shorter, J. (2015) Repurposing Hsp104 to antagonize seminal amyloid and counter HIV infection. *Chem. Biol.* **22**, 1074–1086 <https://doi.org/10.1016/j.chembiol.2015.07.007>
- 45 DeSantis, M.E. and Shorter, J. (2012) Hsp104 drives “protein-only” positive selection of Sup35 prion strains encoding strong [PSI⁺]. *Chem. Biol.* **19**, 1400–1410 <https://doi.org/10.1016/j.chembiol.2012.09.013>
- 46 Guo, L., Kim, H.J., Wang, H., Monaghan, J., Freyermuth, F., Sung, J.C. et al. (2018) Nuclear-import receptors reverse aberrant phase transitions of RNA-binding proteins with prion-like domains. *Cell* **173**, 677–692.e620 <https://doi.org/10.1016/j.cell.2018.03.002>
- 47 Huang, L., Agrawal, T., Zhu, G., Yu, S., Tao, L., Lin, J. et al. (2021) DAXX represents a new type of protein-folding enabler. *Nature* **597**, 132–137 <https://doi.org/10.1038/s41586-021-03824-5>
- 48 Klaiaps, C.L., Hochstrasser, M.L., Langlois, C.R. and Serio, T.R. (2014) Spatial quality control bypasses cell-based limitations on proteostasis to promote prion curing. *eLife* **3**, e04288 <https://doi.org/10.7554/eLife.04288>
- 49 Lo Bianco, C., Shorter, J., Regulier, E., Lashuel, H., Iwatsubo, T., Lindquist, S. et al. (2008) Hsp104 antagonizes alpha-synuclein aggregation and reduces dopaminergic degeneration in a rat model of Parkinson disease. *J. Clin. Invest.* **118**, 3087–3097 <https://doi.org/10.1172/JCI35781>
- 50 Mahapatra, S., Sarbahi, A., Madhu, P., Swasthi, H.M. and Mukhopadhyay, S. (2021) Sub-stoichiometric Hsp104 regulates the genesis and persistence of self-replicable amyloid seeds of a yeast prion protein. *bioRxiv* <https://doi.org/10.1101/2021.03.08.434509>
- 51 Park, Y.N., Zhao, X., Yim, Y.I., Todor, H., Ellerbrock, R., Reidy, M. et al. (2014) Hsp104 overexpression cures *saccharomyces cerevisiae* [PSI⁺] by causing dissolution of the prion seeds. *Eukaryot. Cell* **13**, 635–647 <https://doi.org/10.1128/EC.00300-13>
- 52 Pei, F., DiSalvo, S., Sindi, S.S. and Serio, T.R. (2017) A dominant-negative mutant inhibits multiple prion variants through a common mechanism. *PLoS Genet.* **13**, e1007085 <https://doi.org/10.1371/journal.pgen.1007085>
- 53 Shorter, J. and Lindquist, S. (2004) Hsp104 catalyzes formation and elimination of self-replicating Sup35 prion conformers. *Science* **304**, 1793–1797 <https://doi.org/10.1126/science.1098007>
- 54 Shorter, J. and Lindquist, S. (2006) Destruction or potentiation of different prions catalyzed by similar Hsp104 remodeling activities. *Mol. Cell* **23**, 425–438 <https://doi.org/10.1016/j.molcel.2006.05.042>
- 55 Shorter, J. and Lindquist, S. (2008) Hsp104, Hsp70 and Hsp40 interplay regulates formation, growth and elimination of Sup35 prions. *EMBO J.* **27**, 2712–2724 <https://doi.org/10.1038/emboj.2008.194>
- 56 Villali, J., Dark, J., Brechtel, T.M., Pei, F., Sindi, S.S. and Serio, T.R. (2020) Nucleation seed size determines amyloid clearance and establishes a barrier to prion appearance in yeast. *Nat. Struct. Mol. Biol.* **27**, 540–549 <https://doi.org/10.1038/s41594-020-0416-6>
- 57 Zhu, G., Harischandra, D.S., Ghaisas, S., Zhang, P., Prall, W., Huang, L. et al. (2020) TRIM11 prevents and reverses protein aggregation and rescues a mouse model of Parkinson’s disease. *Cell Rep.* **33**, 108418 <https://doi.org/10.1016/j.celrep.2020.108418>
- 58 Sweeny, E.A., Jackrel, M.E., Go, M.S., Sochor, M.A., Razzo, B.M., DeSantis, M.E. et al. (2015) The Hsp104 N-terminal domain enables disaggregase plasticity and potentiation. *Mol. Cell* **57**, 836–849 <https://doi.org/10.1016/j.molcel.2014.12.021>
- 59 Cushman-Nick, M., Bonini, N.M. and Shorter, J. (2013) Hsp104 suppresses polyglutamine-induced degeneration post onset in a drosophila MJD/SCA3 model. *PLoS Genet.* **9**, e1003781 <https://doi.org/10.1371/journal.pgen.1003781>
- 60 March, Z.M., Mack, K.L. and Shorter, J. (2019) AAA+ protein-based technologies to counter neurodegenerative disease. *Biophys. J.* **116**, 1380–1385 <https://doi.org/10.1016/j.bpj.2019.03.007>
- 61 Duenwald, M.L., Echeverria, A. and Shorter, J. (2012) Small heat shock proteins potentiate amyloid dissolution by protein disaggregases from yeast and humans. *PLoS Biol.* **10**, e1001346 <https://doi.org/10.1371/journal.pbio.1001346>
- 62 Shorter, J. (2011) The mammalian disaggregase machinery: Hsp110 synergizes with Hsp70 and Hsp40 to catalyze protein disaggregation and reactivation in a cell-free system. *PLoS ONE* **6**, e26319 <https://doi.org/10.1371/journal.pone.0026319>
- 63 Ye, X., Mayne, L. and Englander, S.W. (2021) A conserved strategy for structure change and energy transduction in Hsp104 and other AAA+ protein motors. *J. Biol. Chem.* **297**, 101066 <https://doi.org/10.1016/j.jbc.2021.101066>
- 64 Michalska, K., Zhang, K., March, Z.M., Hatzos-Skitnges, C., Pintilie, G., Bigelow, L. et al. (2019) Structure of *Calcarisporiella thermophila* Hsp104 disaggregase that antagonizes diverse proteotoxic misfolding events. *Structure* **27**, 449–463.e447 <https://doi.org/10.1016/j.str.2018.11.001>
- 65 Yu, H., Lupoli, T.J., Kovach, A., Meng, X., Zhao, G., Nathan, C.F. et al. (2018) ATP hydrolysis-coupled peptide translocation mechanism of *Mycobacterium tuberculosis* ClpB. *Proc. Natl Acad. Sci. U.S.A.* **115**, E9560–E9569 <https://doi.org/10.1073/pnas.1810648115>
- 66 Kotamarthi, H.C., Sauer, R.T. and Baker, T.A. (2020) The non-dominant AAA+ ring in the ClpAP protease functions as an anti-stalling motor to accelerate protein unfolding and translocation. *Cell Rep.* **30**, 2644–2654.e2643 <https://doi.org/10.1016/j.celrep.2020.01.110>
- 67 Rajendar, B. and Lucius, A.L. (2010) Molecular mechanism of polypeptide translocation catalyzed by the *Escherichia coli* ClpA protein translocase. *J. Mol. Biol.* **399**, 665–679 <https://doi.org/10.1016/j.jmb.2010.03.061>
- 68 Reid, B.G., Fenton, W.A., Horwich, A.L. and Weber-Ban, E.U. (2001) ClpA mediates directional translocation of substrate proteins into the ClpP protease. *Proc. Natl Acad. Sci. U.S.A.* **98**, 3768–3772 <https://doi.org/10.1073/pnas.071043698>
- 69 Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S. et al. (2004) Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell* **119**, 653–665 <https://doi.org/10.1016/j.cell.2004.11.027>

- 70 Shorter, J. and Lindquist, S. (2005) Navigating the ClpB channel to solution. *Nat. Struct. Mol. Biol.* **12**, 4–6 <https://doi.org/10.1038/nsmb0105-4>
- 71 Cipriano, D.J., Jung, J., Vivona, S., Fenn, T.D., Brunger, A.T. and Bryant, Z. (2013) Processive ATP-driven substrate disassembly by the N-ethylmaleimide-sensitive factor (NSF) molecular machine. *J. Biol. Chem.* **288**, 23436–23445 <https://doi.org/10.1074/jbc.M113.476705>
- 72 Scull, N.W. and Lucius, A.L. (2020) Kinetic analysis of AAA+ translocases by combined fluorescence and anisotropy methods. *Biophys. J.* **119**, 1335–1350 <https://doi.org/10.1016/j.bpj.2020.08.018>
- 73 Olivares, A.O., Nager, A.R., Iosefson, O., Sauer, R.T. and Baker, T.A. (2014) Mechanochemical basis of protein degradation by a double-ring AAA+ machine. *Nat. Struct. Mol. Biol.* **21**, 871–875 <https://doi.org/10.1038/nsmb.2885>
- 74 Maillard, R.A., Chistol, G., Sen, M., Righini, M., Tan, J., Kaiser, C.M. et al. (2011) ClpX(P) generates mechanical force to unfold and translocate its protein substrates. *Cell* **145**, 459–469 <https://doi.org/10.1016/j.cell.2011.04.010>
- 75 Hoskins, J.R., Yanagihara, K., Mizuuchi, K. and Wickner, S. (2002) ClpAP and ClpXP degrade proteins with tags located in the interior of the primary sequence. *Proc. Natl Acad. Sci. U.S.A.* **99**, 11037–11042 <https://doi.org/10.1073/pnas.172378899>
- 76 Schlieker, C., Weibezahn, J., Patzelt, H., Tessarz, P., Strub, C., Zeth, K. et al. (2004) Substrate recognition by the AAA+ chaperone ClpB. *Nat. Struct. Mol. Biol.* **11**, 607–615 <https://doi.org/10.1038/nsmb787>
- 77 Li, T., Lin, J. and Lucius, A.L. (2015) Examination of polypeptide substrate specificity for *Escherichia coli* ClpB. *Proteins*. **83**, 117–134 <https://doi.org/10.1002/prot.24710>
- 78 Haslberger, T., Zdanowicz, A., Brand, I., Kirstein, J., Turgay, K., Mogk, A. et al. (2008) Protein disaggregation by the AAA+ chaperone ClpB involves partial threading of looped polypeptide segments. *Nat. Struct. Mol. Biol.* **15**, 641–650 <https://doi.org/10.1038/nsmb.1425>
- 79 Yoo, H., Bard, J.A.M., Piliipenko, E.V. and Drummond, D.A. (2022) Chaperones directly and efficiently disperse stress-triggered biomolecular condensates. *Mol. Cell* **82**, 741–755.e711 <https://doi.org/10.1016/j.molcel.2022.01.005>
- 80 Fei, X., Bell, T.A., Barkow, S.R., Baker, T.A. and Sauer, R.T. (2020) Structural basis of ClpXP recognition and unfolding of ssrA-tagged substrates. *eLife* **9**, e61496 <https://doi.org/10.7554/eLife.61496>
- 81 Ripstein, Z.A., Vahidi, S., Houry, W.A., Rubinstein, J.L. and Kay, L.E. (2020) A processive rotary mechanism couples substrate unfolding and proteolysis in the ClpXP degradation machinery. *eLife* **9**, e52158 <https://doi.org/10.7554/eLife.52158>
- 82 Veronese, P.K., Stafford, R.P. and Lucius, A.L. (2009) The *Escherichia coli* ClpA molecular chaperone self-assembles into tetramers. *Biochemistry* **48**, 9221–9233 <https://doi.org/10.1021/bi900935q>
- 83 Lin, J. and Lucius, A.L. (2016) Examination of ClpB quaternary structure and linkage to nucleotide binding. *Biochemistry* **55**, 1758–1771 <https://doi.org/10.1021/acs.biochem.6b00122>
- 84 Han, H., Fulcher, J.M., Dandey, V.P., Iwasa, J.H., Sundquist, W.I., Kay, M.S. et al. (2019) Structure of Vps4 with circular peptides and implications for translocation of two polypeptide chains by AAA+ ATPases. *eLife* **8**, e44071 <https://doi.org/10.7554/eLife.44071>
- 85 van den Boom, J., Kueck, A.F., Kravic, B., Muschenborn, H., Giesing, M., Pan, D. et al. (2021) Targeted substrate loop insertion by VCP/p97 during PP1 complex disassembly. *Nat. Struct. Mol. Biol.* **28**, 964–971 <https://doi.org/10.1038/s41594-021-00684-5>
- 86 San Martin, A., Rodriguez-Aliaga, P., Molina, J.A., Martin, A., Bustamante, C. and Baez, M. (2017) Knots can impair protein degradation by ATP-dependent proteases. *Proc. Natl Acad. Sci. U.S.A.* **114**, 9864–9869 <https://doi.org/10.1073/pnas.1705916114>
- 87 Sivertsson, E.M., Jackson, S.E. and Itzhaki, L.S. (2019) The AAA+ protease ClpXP can easily degrade a 31 and a 52-knotted protein. *Sci. Rep.* **9**, 2421 <https://doi.org/10.1038/s41598-018-38173-3>
- 88 Yin, Y., Feng, X., Yu, H., Fay, A., Kovach, A., Glickman, M.S. et al. (2021) Structural basis for aggregate dissolution and refolding by the *Mycobacterium tuberculosis* ClpB-DnaK bi-chaperone system. *Cell Rep.* **35**, 109166 <https://doi.org/10.1016/j.celrep.2021.109166>
- 89 Alberty, R.A. (1969) Standard Gibbs free energy, enthalpy, and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates. *J. Biol. Chem.* **244**, 3290–3302 [https://doi.org/10.1016/S0021-9258\(18\)93127-3](https://doi.org/10.1016/S0021-9258(18)93127-3)
- 90 Lohman, T.M. and Bjornson, K.P. (1996) Mechanisms of helicase-catalyzed DNA unwinding. *Annu. Rev. Biochem.* **65**, 169–214 <https://doi.org/10.1146/annurev.bi.65.070196.001125>
- 91 Simmons, R.M. and Hill, T.L. (1976) Definitions of free energy levels in biochemical reactions. *Nature* **263**, 615–618 <https://doi.org/10.1038/263615b0>
- 92 Mazal, H., Ilijina, M., Riven, I. and Haran, G. (2021) Ultrafast pore-loop dynamics in a AAA+ machine point to a Brownian-ratchet mechanism for protein translocation. *Sci. Adv.* **7**, eabg4674 <https://doi.org/10.1126/sciadv.abg4674>
- 93 Murray, A.N. and Kelly, J.W. (2012) Hsp104 gives clients the individual attention they need. *Cell* **151**, 695–697 <https://doi.org/10.1016/j.cell.2012.10.033>
- 94 Lin, J. and Lucius, A.L. (2015) Examination of the dynamic assembly equilibrium for *E. coli* ClpB. *Proteins* **83**, 2008–2024 <https://doi.org/10.1002/prot.24914>
- 95 Oguchi, Y., Kummer, E., Seyffer, F., Berynsky, M., Anstett, B., Zahn, R. et al. (2012) A tightly regulated molecular toggle controls AAA+ disaggregase. *Nat. Struct. Mol. Biol.* **19**, 1338–1346 <https://doi.org/10.1038/nsmb.2441>
- 96 DeSantis, M.E., Sweeny, E.A., Snead, D., Leung, E.H., Go, M.S., Gupta, K. et al. (2014) Conserved distal loop residues in the Hsp104 and ClpB middle domain contact nucleotide-binding domain 2 and enable Hsp70-dependent protein disaggregation. *J. Biol. Chem.* **289**, 848–867 <https://doi.org/10.1074/jbc.M113.520759>