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Hsp104 and potentiated variants can operate as distinct non-processive translocases

Clarissa L. Durie\textsuperscript{1,3}, JiaBei Lin\textsuperscript{2}, Nathaniel W. Scull\textsuperscript{1}, Korrie L. Mack\textsuperscript{2}, Meredith E. Jackrel\textsuperscript{1,4}, Elizabeth A. Sweeny\textsuperscript{2, 5}, Laura M. Castellano\textsuperscript{2}, James Shorter\textsuperscript{2}, and Aaron L. Lucius\textsuperscript{1*}

\textsuperscript{1}University of Alabama at Birmingham, Chemistry Department, Birmingham, AL

\textsuperscript{2}Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

\textsuperscript{3}Present address: Life Sciences Institute, University of Michigan, Ann Arbor, MI

\textsuperscript{4}Present address: Department of Chemistry, Washington University, St. Louis, MO

\textsuperscript{5}Present address: Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH

*Correspondence:
Aaron L. Lucius
allucius@uab.edu
Abstract
Hsp104 is a hexameric AAA+ motor protein that enables cells to survive extreme stress. Hsp104 couples the energy of ATP binding and hydrolysis to solubilize proteins trapped in aggregated structures. The mechanism by which Hsp104 disaggregates proteins is not completely understood but may require Hsp104 to partially or completely translocate polypeptides across its central channel. Here, we apply transient state, single turnover kinetics to investigate ATP-dependent translocation of soluble polypeptides by Hsp104 and Hsp104\(^{A503S}\), a potentiated variant developed to resolve misfolded conformers implicated in neurodegenerative disease. We establish that Hsp104 and Hsp104\(^{A503S}\) can operate as nonprocessive translocases for soluble substrates, indicating a “partial threading” model of translocation. Remarkably, Hsp104\(^{A503S}\) exhibits altered coupling of ATP binding to translocation and decelerated dissociation from polypeptide substrate compared to Hsp104. This altered coupling and prolonged substrate interaction likely increase entropic pulling forces thereby enabling more effective aggregate dissolution by Hsp104\(^{A503S}\).

Introduction
Hsp104 rescues damaged proteins from aggregates, helping organisms survive and adapt to environmental stresses (1, 2). A member of the AAA+ (ATPases associated with diverse cellular activities) protein family, Hsp104 uses the energy of ATP binding and hydrolysis to perform mechanical work (3). Hsp104 and its homologues are conserved through bacteria, fungi, protozoa, algae, and plants, but no such homologue exists in the cytosol of animal cells (4, 5). Because protein aggregates and toxic soluble oligomers are implicated in many neurodegenerative diseases, there is interest in developing therapeutic forms of Hsp104 that can safely dissociate soluble toxic oligomers and reverse aggregation associated with neurodegenerative diseases (6). Recently, potentiated variants of yeast Hsp104 were shown to rescue yeast, C. elegans, and mammalian cell models of human neurodegenerative diseases (7-9). However, many questions remain open regarding how, at the molecular level, both the wild-type (WT) disaggregase and potentiated variants catalyze protein disaggregation.

A “complete threading” translocation mechanism is often advanced as describing Hsp104-catalyzed protein disaggregation. In this model, ATP binding and hydrolysis are coupled to translocating the entire polypeptide across the axial channel (10, 11). This model is based largely on structural similarities between Hsp104 and E. coli ClpA, another Hsp100 and AAA+ family member that also works in protein-quality control (4). ClpA couples the energy from ATP binding and hydrolysis to processive translocation of proteins through the axial channel of the ClpA homohexameric ring, into the proteolytic partner protein ClpP (12-16).

Notably, although Hsp104 has been connected to degradation of select substrates (17, 18) it typically promotes protein solubilization and not degradation (1, 19) and, natively, is not able to bind ClpP (20). Experimental evidence in support of a complete threading model for Hsp104 comes largely from an engineered interaction with ClpP. Hsp104 can be engineered to interact with ClpP by introducing three mutations (G739I:S740G:K741F) based on ClpA into a helix-loop-helix motif (20). The resulting Hsp104 variant, termed HAP, is able to promote degradation
of soluble and aggregated polypeptides in the presence of ClpP, including stable amyloid fibrils (20, 21). These findings are consistent with complete translocation. However, studies with the E. coli ClpB version of HAP, termed BAP (in which S722-N748 of ClpB are replaced with V609-I635 of ClpA (22)), indicate that BAP-ClpP can degrade substrates in an ATP-independent manner (23). Thus, it continues to remain uncertain whether ClpB catalyzes processive translocation of the protein substrate driven by ATP hydrolysis.

Recent high-resolution structural data has also been interpreted in the context of a threading model (24, 25). Cryo-EM reconstructions have revealed Hsp104 in closed and extended right-handed spiral states bound to casein in the presence of ATPγS, as well as in left-handed open ‘lock-washer’ states in the presence of AMP-PNP or ADP and absence of a protein substrate (24, 25). By morphing these images, a ratcheting mechanism of polypeptide translocation was proposed (24, 25). A conformational switch from the open to closed state is proposed to bring polypeptide into the channel in an ATP-hydrolysis independent step, thereafter the hexamer might switch between successive, energy driven, closed and extended states to further translocate polypeptide processively into the Hsp104 channel (24, 25). Consistently, in other studies, ClpB or an ATPase-dead variant of BAP, BAP E279A/E678A, was observed with casein deep (~70-80Å) in the axial channel (26, 27). This observation suggests, at least in the case of BAP, ATP hydrolysis is not required for engaging substrate into the channel. However, this observation is not sufficient to support that casein is deeply “translocated” into the central pore. Another possibility is that the hexameric ring binds by wrapping around the protein substrate, resulting in the substrate deep within the axial channel without ATP dependent translocation. Here, we seek to test the mechanism by which Hsp104 and potentiated variants translocate substrates, in solution, using transient state kinetic techniques.

Though “complete threading” and “partial threading” are commonly used in describing the unfolding, disaggregating, or translocating activities of Hsp104, how these descriptions might relate to processivity is uncertain. Here, we will use a mathematical definition of processivity (28). We define processivity as the probability that the enzyme will either step forward with translocation rate constant $k_t$ or dissociate with dissociation rate constant $k_d$. This definition is illustrated in Scheme 1 for an arbitrary enzyme-substrate complex, $ES$, proceeding forward with rate constant $k_t$ to form the first translocation intermediate, $I_1$, or dissociate with rate constant $k_d$ into free enzyme, $E$, and free substrate, $S$. For a highly processive enzyme, this cycle would repeat multiple times before reaching the end of the lattice, assuming the lattice is much longer than the step-size.
The processivity is mathematically defined by Equation (1)

\[ \text{Pr} = \frac{k_i}{k_i + k_d} \]  

(1)

where \( \text{Pr} \) is processivity and, \( k_i \) and \( k_d \) are defined above. As \( k_d \) approaches zero, processivity approaches unity. This case would describe an infinitely processive enzyme, again, assuming the lattice is much longer than the step-size thereby allowing for the possibility of multiple steps (12, 29, 30). In contrast, as \( k_d \) exceeds \( k_i \) then \( \text{Pr} \) approaches zero, describing a distributive enzyme. Thus, the processivity, \( \text{Pr} \), as defined by Equation (1) is a probability that varies between zero and one. Processivity can also be defined as the average number of lattice elements translocated per binding event. For a protein translocase the lattice element would be amino acids. The probability processivity, \( \text{Pr} \), can be related to a processivity, \( N \), expressed as the average number of amino acids translocated per binding event through Equation (2),

\[ \text{Pr} = \frac{k_i}{k_i + k_d} = e^{(-m/k_i)} \]  

(2)

where \( m \) represents the step-size as the distance translocated in amino acids per step. With these definitions in mind, the model that Hsp104 completely threads a polypeptide chain through its axial channel is regarded as indicating a processive enzyme.

The “complete threading” model was widely accepted for the bacterial homologue of Hsp104, *E. coli* ClpB. Like Hsp104, ClpB has high structural similarity with ClpA. Thus, Bukau and co-workers employed a similar protein-engineering strategy to observe substrate degradation by a
variant of ClpB, termed BAP (ClpB with ClpA’s P-loop). In this construct, ClpB S722-N748 was replaced with V609-I635 of ClpA; thereby, engineering ClpB to bind the protease ClpP (22, 31). Recent transient state kinetic analysis of ClpB-catalyzed, ATP-dependent translocation of a polypeptide substrate revealed, however, that ClpB is a non-processive polypeptide translocase (23). Rather than completely threading an entire polypeptide chain through its axial channel, ClpB was observed to take only one or two kinetic steps on a polypeptide substrate independent of the length of the polypeptide chain. Using Equation (2) with \( N = 1 \text{m} \) or \( N = 2 \text{m} \) predicts a processivity \( Pr = 0.37 \) and 0.61 for one step or two steps, respectively. This led us to propose a “tug and release” mechanism by which ClpB can resolve an aggregate by taking one or two translocation steps before dissociation and subsequent rebinding to repeat the cycle (23).

Consistent with this conclusion, ClpB and Hsp104 have been reported to use a “partial threading” mechanism to disaggregate some substrates (32). Moreover, Hsp104 employs a partial translocation mechanism to dissolve Sup35 prions (3, 33). Thus, an open question is whether Hsp104 and ClpB typically disaggregate substrates via processive or nonprocessive translocation or via both mechanisms.

Here, we test predictions of the recent high-resolution structural investigation of Hsp104 and its translocation mechanism. To do so, we conduct an in-solution investigation of the Hsp104 mechanism of ATP-dependent polypeptide translocation. Furthermore, to better understand how translocation and disaggregation are coupled we investigate how the mechanism of the potentiated variant Hsp104\(^{A503S}\) differs from Hsp104. Using soluble polypeptide substrates, we establish that Hsp104 and Hsp104\(^{A503S}\) proceed through only one or two kinetic steps before dissociation from the soluble polypeptide chain, independent of chain length. This observation is inconsistent with “complete threading” by taking many steps of a small size (e.g. two amino acids) or highly processive translocation of the soluble polypeptide chain. Strikingly, however, we observe that the A503S mutation in the middle domain of Hsp104 alters the coupling of ATP binding to the observed translocation steps. Indeed, Hsp104\(^{A503S}\) exhibits decelerated dissociation from soluble polypeptide substrate compared to Hsp104. This altered mode of translocation likely increases entropic pulling forces thereby enabling more effective aggregate dissolution by Hsp104\(^{A503S}\).

**Methods**

*Buffers and Reagents*

All buffers were prepared with distilled, deionized water from a Purelab Ultra Genetic system (Evoqua, Warrendale, PA). All chemicals were reagent grade. ATP\(^{\gamma}\)S was purchased from CalBiochem (La Jolla, Ca).

All experiments were performed in buffer HK150. Buffer HK150 contains 25 mM HEPES, 150 mM KCl, 10 mM MgCl, 2 mM DTT, and 10% glycerol (v/v), with pH 7.5 at 25 °C.

*Proteins and Peptides*
Hsp104 and Hsp104A503S were prepared as previously described.(7, 34, 35) The concentration of Hsp104 was determined at 280 nm in HK150 using the extinction coefficient $\varepsilon = 32,500 \text{ M}^{-1}\text{cm}^{-1}$, determined following the method of Edelhoch.(36, 37) All protein concentrations are reported in monomer units.

RepA truncation peptides were prepared by CPC Scientific (Sunnyvale, CA). The peptides are made of the first 30, 40, or 50 amino acids of the N terminal sequence of RepA, with a cysteine residue added to either the N or C terminus for labeling. Labeling with fluorescein-5-maleimide was performed as previously described, forming a covalent bond between the peptide and the fluorophore.(38) $\alpha$S1–casein truncation peptides were prepared and labeled with fluorescein as previously described.(39)

**Steady State Fluorescence Quenching Control**

Total fluorescence of RepA$_{1-30}$Flu (100 nM final concentration) was observed in a Fluorolog-3 spectrophotometer (HORIBA Jobin Yvon, Edison, NJ) at 25 °C. Fluorescence was excited at 494 nm and an emission was collected from 500-600 nm. ATP$_{\gamma}$S was added (final concentration of 300 µM) and allowed to incubate for approximately 20 minutes, after which time an emission spectrum was collected. Finally, Hsp104 (final concentration of 2 µM) was then added, allowed to incubate for 20 minutes, and another fluorescence emission spectrum was collected. Upon addition of each component, fluorescence cuvettes were gently inverted to mix the reaction solution. All reagents were equilibrated to 25 °C for approximately one hour prior to addition to the fluorescence cuvette. Simultaneous anisotropy and total fluorescence measurements were also collected (with excitation at 494 nm and emission at 515 nm) to confirm that fluorescence quenching was due to binding of RepA$_{1-30}$Flu by Hsp104 in the presence of ATP$_{\gamma}$S (data not shown).

**Rapid Mixing Experiments with Fluorescein-Modified RepA Truncation Peptides**

All rapid mixing experiments were performed using an Applied Photophysics SX20 stopped flow fluorometer. Fluorescence of fluorescein-modified peptides were excited at 494 nm and observed using a 515 nm cut off long pass filter. All solutions were incubated for 1 hour at 25 °C prior to mixing. The syringes of the stopped-flow fluorimeter and the reaction cell were also maintained at 25 °C. For each concentration of ATP, a minimum of 6 shots or pushes were collected and averaged. Each time course was at least 5 half-lives of the slowest kinetic step.

**Substrate length dependence**

One syringe of the stopped flow fluorometer contained a solution of RepA peptide (100 nM), Hsp104 (2 µM), and ATP$_{\gamma}$S (300 µM). The other syringe contained a solution of 10 mM ATP and 20 µM $\alpha$-casein. The solutions were loaded, separately, into the stopped flow fluorimeter. The solutions were rapidly mixed. Each experiment was performed in triplicate. Experiments were conducted in the same manner for Hsp104A503S, except that only NCys-labeled RepA peptides were used.

**[ATP] dependence**
A solution of RepA_Fha1-50 (20 nM), Hsp104 (2 µM), and ATPγS (300 µM) was incubated in one syringe of the stopped flow fluorimeter. A solution of ATP (various concentrations) and 20 µM α-casein was incubated in the other syringe. The solutions were rapidly mixed. Each experiment was performed in triplicate.

**Rapid Mixing Experiments with Fluorescein-Modified α-Casein Truncation Peptides**

Experiments were performed using an Applied Photophysics SX20 stopped flow fluorometer as described above with the additional use of the Fluorescence Polarisation Accessory. Two R-6095 photomultiplier tubes were used, each with a 515 nm cut off long pass filter. One syringe of the stopped flow fluorometer contained a solution of fluorescein labeled α-casein peptide (20 nM), Hsp104 (2 µM), and ATPγS (300 µM). The other syringe contained a solution of 10 mM ATP and 20 µM α-casein. The solutions were loaded, separately, into the stopped flow fluorimeter. The solutions were rapidly mixed. Experiments were replicated.

**NLLS Analysis of Transient State Kinetics**

See supplemental NLLS Analysis of Transient State Kinetics for derivations pertaining to the models used for data analysis.

**Results**

**Experimental logic and design**

Recent structural data resulted in the proposal of a processive rotary translocation, or ratcheting, mechanism for Hsp104-catalyzed translocation of peptide substrate. In this model, conformational changes in Hsp104 protomers are coupled to ATP binding and hydrolysis. These conformational changes were proposed to advance the interactions between the pore loops of the Hsp104 hexamer and the engaged peptide by two amino acids per step. In this model, the movement of the peptide proceeds from Hsp104 Nucleotide Binding Domain (NBD)1 to NBD2 (25).

Here, we test the proposed structure-based model using transient state kinetics. The experiments presented here are specifically designed to determine the number of ATP driven steps the disaggregase takes before dissociation from the model polypeptide chain. In this approach, we use stopped-flow fluorescence to monitor the ATP-dependent peptide translocation and dissociation of Hsp104, as we have done with other AAA+ translocases (12, 13, 23).

Figure 1A is a schematic representation of Hsp104 and the primary structural features are labeled as the N-terminal domain (NTD), NBD1, middle (M) domain, and NBD2. Figure 1B schematically depicts the method. In this design, Hsp104 is bound to a fluorescently labeled peptide (See Table 1) in the presence of ATPγS, where the ATPγS is included to ensure avid binding to the polypeptide substrate (25, 38). The preformed complex always exhibits a fluorescence quenching when Hsp104 is bound (Supplemental Figure 1) and thus a recovery in fluorescence when Hsp104 dissociates. This preformed and fluorescently quenched complex is rapidly mixed with ATP and a protein trap. In this experimental design, the hydrolysable ATP provides the energy for translocation and the protein trap binds any Hsp104 not bound to fluorescently labeled peptide or that dissociates from the polypeptide during or after
translocation. The trap ensures that the signal reports on a single turnover of the Hsp104:Flu- peptide complex through multiple rounds of ATP binding and hydrolysis. It is important to note that we cannot distinguish between dissociation of a hexamer as a single unit vs. dissociation through disassembly of the hexamer. However, the trap ensures that if dissociation is the result of disassembly then reassembly on the polypeptide is not possible.

Although ATPγS will be slowly hydrolyzed by Hsp104 in the pre-incubation syringe, we have no evidence that this hydrolysis of ATPγS can be coupled to translocation beyond encapsulating substrate in the channel as observed via cryo-EM (25-27). Indeed, we observe no ‘shot-to-shot’ variability over several hours, where shot-to-shot variability would indicate motor movement during the pre-incubation period (data not shown).

**Simulated time courses for Hsp104-driven polypeptide translocation**

It is unknown where Hsp104 binds on the polypeptides used in this study (see Table 1). Also unknown is the directionality of translocation relative to the C- or N-terminus of the polypeptide chain. However, peptide is thought to enter the axial channel of Hsp104 at NBD1 and is translocated to NBD2 (Figure 1A) (25). Thus, the directionality with respect to Hsp104 is assumed to be from NBD1 to NBD2. However, it is unclear if the motor translocates from N- to C-terminus or from C- to N-terminus on the polypeptide lattice. Evidence from other substrates suggests that Hsp104 can translocate in either direction, from N- to C-terminus or from C- to N-terminus (40, 41).

To simulate the expected signals from the kinetic time courses acquired from the stopped-flow experiment schematized in Figure 1B we have schematized the potential sites on the polypeptide chain where Hsp104 can initiate translocation. Figure 1C assumes the N-terminus of the polypeptide chain is bound by Hsp104, enters Hsp104 at NBD1, and is translocated toward NBD2. In contrast, Figure 1D assumes the C-terminus of the peptide is bound by Hsp104, again entering from NBD1 of Hsp104. In both Figure 1C and D the green star represents the position of the fluorophore and when it is not visible it is covered by Hsp104. Thus, the peptides are fluorescently modified at either the N- or C-terminus.

In the presence of ATPγS, based on the structural data, Hsp104 is initially bound to polypeptide substrate in the closed conformation with 26 amino acids of the substrate already inside the axial channel (25). Upon initiation of ATP-driven translocation Hsp104 is proposed to step along the polypeptide chain with a step size of 2 amino acids per step (25). Our experimental design is not sensitive to the open conformation of the Hsp104 hexamer, which is not populated in the presence of ATPγS and polypeptide substrate (24, 25).

With the above structural model in mind, we sought to simulate expected time courses for each of the potential starting positions and dye positions indicated in Figure 1C–H. We consider representative possibilities in which Hsp104 translocates the entire length of a peptide substrate, some partial length of the peptide, or only the length of the peptide that is initially bound by the disaggregase.
In our method we use a set of three polypeptides of length \( L \) 30, 40, and 50 amino acids. If Hsp104 is bound to either the N- or C-terminus as illustrated in Figure 1C and D then 4, 14, and 24 amino acids would extend out of the axial channel (i.e. \( L - 26 \)).

For Figure 1C and D, the structural model that proposed 2 amino acids per step predicts Hsp104 would take 15, 20, and 25 steps on the 30, 40 and 50 amino acid substrates, respectively for complete translocation. The kinetic time courses that we predict for these three substrates for the binding conditions shown in Figure 1C–D are shown in Supplemental Figure 2A. If Hsp104 is proceeding through ~15, 20, and 25 steps, the time courses are predicted to show a strong length dependence regardless of position of the fluorescent probe since, in this assay, fluorescence is constant until dissociation.

Figure 1E and F illustrate the possibility that Hsp104 may bind in the middle of a polypeptide chain. As with binding at either the N- or C-terminus, the structural model predicts that, when bound in the middle, Hsp104 would still occlude 26 amino acids (25). In this case, the number of amino acids remaining outside of the channel on either end of the Hsp104 binding site would be fewer than when the disaggregase is bound to either the N- or C-terminus, i.e. 2, 7, and 12 amino acids for the 30, 40, and 50 amino acid substrates, respectively ((\( L - 26 \))/2). Processive unidirectional translocation would then require the motor to translocate 28, 33, and 38 amino acids, respectively. Thus, for the binding condition illustrated by Fig. 1E – F, we predict 14, 16.5, and 19 steps on the 30, 40, and 50 amino acid substrates, respectively. Supplemental Figure 2B shows the predicted time courses for the 30, 40 and, 50 amino acid substrates initiating ATP driven translocation from the middle as illustrated in Figure 1E – F. Similar to binding at the end, the simulated time courses predict a clear substrate length dependence (Supplemental Figure 2B).

Figure 1G and H illustrate Hsp104 binding to either the N- or C-terminus with the polypeptide extruding from the NBD2 side of the disaggregase. In this arrangement Hsp104 would again occlude 26 amino acids and proceed through 13 steps with a 2 amino acid step size. However, because the disaggregase is bound to the end of the polypeptide and translocating the substrate from NBD1 to NBD2 we predict a length independent lag. That is to say, regardless of substrate length, we predict that Hsp104 would take the same number of steps. Thus, the time course would exhibit a lag, which would be the same regardless of substrate length as illustrated in supplemental Figure 2C. However, this possibility does not agree with the Cryo-EM observation of substrate extending from the NBD1 end and not the NBD2 end of the Hsp104 hexamer (25). Note that recent CryoEM studies of ClpB and ATPase-dead BAP also revealed protein substrate occupancy in NBD1 but not NBD2 (26, 27). Thus, while this possibility cannot be excluded, it is not supported by current structural data (25-27). If the CryoEM observations represent all possible arrangements of Hsp104 on substrate, then substrate extending only from NBD1 and not NBD2 must result from incomplete translocation.

It is also possible, perhaps even likely, that Hsp104 protein binds randomly along the polypeptide substrate. In other words, a stochastic distribution of the potential binding sites as illustrated in Figure 1C–H. This scenario would be identical to that of single-stranded DNA translocases that exhibit random binding along the nucleic acid (42, 43). Length-dependent time
courses are observed in an analogous single-turnover experiment. From those simulations we predict that if Hsp104 exhibits random binding along the polypeptide lattice then we would again observe length dependent time courses.

**Experimental assessment of Hsp104-driven polypeptide translocation**

To test the possibilities illustrated in Figure 1 C – H we performed single turnover polypeptide translocation experiments as illustrated in Figure 1B. Upon rapid mixing of the components, fluorescein is excited at 494 nm and emission is observed at and above 515 nm (see Methods). As seen in Figure 2A, the initial fluorescence is low due to quenching in the bound complex (see Supplementary Fig. 1B) and increases over time as Hsp104 dissociates and the fluorescence quenching is relieved. This finding is consistent with observations from the same experimental design studying ClpA and ClpB (12, 23, 38). (See Supplementary Figure 3)

These experiments were carried out with 2 µM monomer Hsp104 and 100 nM peptide. Although this represents a 20-fold excess of Hsp104 monomers the maximum amount of hexamer present and available to bind to peptide is 333 nM. However, as we have previously shown, Hsp104 is in a dynamic equilibrium of hexamers and smaller oligomers (38). Thus, the actual concentration of hexamers is lower. We have performed experiments with 1 µM and 200 nM Hsp104 and the kinetic parameters are comparable (see Supplementary Table 1).

Figure 2A (solid traces) displays representative time courses from the Hsp104-peptide complex prepared with RepAFlu1-30, RepAFlu1-40, and RepAFlu1-50 (polypeptides summarized in Table 1). Strikingly, each time course exhibits a lag followed by an increase in signal. This lag indicates that more than one step with similar rate constants occur prior to dissociation from the polypeptide chain, which is consistent with ATP-driven translocation. Moreover, no lag was observed in identical experiments where ATP was left out and those time courses proceed on a longer time scale (see Supplementary Figure 3). The lag observed with Hsp104 contrasts with observations made with ClpB, which exhibits no lag prior to dissociation (23). If Hsp104 is a processive translocase, ratcheting the polypeptide substrate through the axial channel with successive rounds of ATP hydrolysis, this lag is predicted to increase with increasing substrate length as shown in our simulations (Supplemental Figure 2A, B) (12, 13, 29, 30, 44-47). However, Fig. 2A shows that the extent of the lag is the same for all three polypeptide substrates ranging from 30 to 50 amino acids. This observation indicates that, regardless of length, Hsp104 dissociates after taking the same number of steps, which is inconsistent with a motor that completely threads a polypeptide chain through the axial channel by taking many steps of a small size (e.g. two amino acids). This observation may be consistent with the scenario depicted in Figure 1G-H in which Hsp104 binds at the end of the peptide substrate and translocates directly off that end, but this model appears inconsistent with available cryo-EM data (25).

The other prominent feature of the time courses in Fig. 2A is that they are biphasic, which indicates that there are two starting populations proceeding through the same kinetic path (29, 30). The simplest mechanism that accounts for the observed lag and the biphasic nature of the time courses in Fig. 2A is given by Scheme 2.

\[
C^* \xrightarrow{k_{pr}} C \xrightarrow{k_{i}} I_1 \xrightarrow{k_{i}} I_2 \ldots \xrightarrow{k_{i}} I_n \xrightarrow{k} P
\]
Scheme 2

In Scheme 2, C and C* are two conformations of Hsp104-peptide complex in equilibrium. C* is the Hsp104-peptide complex that must first proceed through a step with rate constant $k_{np}$ to form C, which represents Hsp104 bound to peptide in a form that can proceed through ATP-driven translocation steps. The concentrations of these two species are accounted for by the fraction of productively bound complexes, $x$, and thus the fraction of nonproductively bound complexes would be given by $(1-x)$. This step with rate constant $k_{np}$ accounts for the biphasic character of the kinetic time courses. Next, to account for the lag in the kinetic time course, C can proceed through $n$ number of steps with rate constant $k$, before dissociation into $P$, see Methods (Eq 2).

In the context of the mechanism proposed by Gates et al. (25), C* and C could represent two conformations of the ATPγS bound “closed” conformation observed by cryo-EM, with $k_{np}$ representing the conformational change for that transition. Then, $k$, may represent the ratcheting steps that advance the peptide by 2 amino acids through the axial channel of Hsp104 hexamers. While the current assay does not allow attribution of the observed kinetic phases to specific mechanical movements, the observations here do not conflict with the mechanistic model proposed by Gates et al (25).

The time courses shown in Figure 2A (solid traces) were individually fit to the expression derived from Scheme 2 (see Supplemental Methods). Best fit lines are shown as broken black traces in Figure 2A. The parameters from these fits are summarized in the first three rows of Table 2 (mean ± s. e., 3 replicates). Note that these time courses were not well described by a simple sum of exponential equations due to the observed lag (fit not shown). The values for the parameters $k_n$, $n$, and $x$, where $x$ is the fraction of starting complex in the C conformation, exhibited no discernable length-dependent trends and are summarized in the first three rows of Table 2.

The values of $k_{np}$ were noted to vary such that faster rate constants are observed with shorter peptides. One possible explanation has to do with binding location or environment. If 26 amino acids are inside the channel as reported by Gates et al, then there are fewer points of contact for the 30mer compared to the 50mer. If Hsp104 is more likely to bind at or near an end on the 30mer, and it more rapidly transitions from a nonproductive to a productive complex upon mixing with ATP then it is possible that binding positions nearer the end of the peptide favor transitions to the productive complex compared to binding positions in the center of the peptide.

The average and standard error of the number of steps, $n$, from individual NLLS fits of the time courses in Figure 2A to Scheme 2 are shown in Figure 2B. The number of repeating steps $n$ taken by Hsp104 is constant across the lengths of the substrate tested for Hsp104, with the average $n = (1.6 ± 0.1)$ steps. As noted above, a lag results from the observation of more than one step with similar rate constants. For comparison, the number of steps taken by the processive translocase ClpA varied linearly from $n = 1.4$ steps to 3.0 steps in an identical experiment (12).

If these steps represent repetitions of a 2 amino acid translocation step, then the extent of the lag would be predicted to vary with substrate length for processive translocation (12, 13, 29, 30, 44-47). The formal possibility described in Figure 1 G and H cannot be ruled out from this
experiment, however the simulations predict 13 steps when 26 amino acids are occluded while we observe only two repeated steps. For the scenario illustrated in Figure 1G-H to give rise to these results, only the terminal four residues of the substrate would be bound in the Hsp104 hexamer, with the remainder of the peptide substrate extending from the NDB2 end of the Hsp104 hexamer. This possibility is not supported by structural work showing bound peptides extending from the NBD1 end of the Hsp104 hexamer (25).

Thus, we are left with one of two explanations. Either Hsp104 exhibits low processivity, i.e. the disaggregase takes one or two steps before dissociation independent of length and binding position. Alternatively, translocation is very fast relative to the 1-2 steps we detect in this experiment.

A sufficiently long substrate will exhibit a length dependence even if translocation is fast relative to the two steps we detect using the relatively short RepA substrates. Thus, we next used longer substrates, namely fluorescein-labeled truncations of α-casein, which were 102 and 127 amino acids in length. α-casein has been used as a substrate previously to examine Hsp104 function (7, 20, 25). We have previously used these truncations of α-casein to examine the binding of both ClpA and ClpB. We showed that 127 amino acids and shorter would accommodate only one hexamer of either ClpA or ClpB whereas longer constructs could accommodate two hexamers (39, 48). We applied the same experimental design schematized in Figure 1B with the exception that anisotropy was detected instead of fluorescence. Fluorescence anisotropy was used because it reflects the resident time of the motor on the substrate regardless of directionality (see Methods). As observed with the 30-50 amino acid substrates, Figure 2C shows a lag in the Hsp104-catalyzed translocation and dissociation time course for both the 102 and 127 amino acid substrates, and the lengths of the lags are indistinguishable. In contrast to the observations with Hsp104, the processive translocase ClpA exhibits a length-dependent lag in the same experimental design (Supplementary Figure 5), as previously reported (23).

Anisotropy time courses are influenced by both anisotropy and fluorescence quantum yield. Consequently, the kinetic time courses require a simultaneous analysis of total fluorescence and anisotropy. We subjected the anisotropy time courses and their corresponding total fluorescence time courses to NLLS analysis using the strategy described by Otto et al (49). The time courses for α-casein 102 and 127 were both well described by a model with two kinetic steps (see Supplemental Figure 4 and corresponding supplemental methods). This observation is consistent with Hsp104 taking 1–2 translocation steps even on substantially longer polypeptide substrates compared to the RepA peptides and is not consistent with complete threading or highly processive translocation.

**Directionality of Hsp104-driven polypeptide translocation**

We tested whether Hsp104 displayed a directional bias in its translocation of peptide substrates. Though length-dependence was not observed for N-labeled peptides (Figure 2A-C), we tested whether C-labeled peptides reveal length-dependent time courses. In a previous study using this experimental design, the position of the fluorophore relative to the ClpA binding site yielded information on directionality (12). The RepA truncation peptides and the 127 amino acid casein truncation peptide were prepared with the label at the C-terminus (see Table 1) and tested using
the same experimental designs. Again, a lag was observed in the time courses from the 30, 40, and 50 amino acid peptides and, again, the number of steps taken was independent of substrate length (Supplemental Figure 6A; Supplemental Table 2). Additionally, the anisotropy time courses of the N-labeled and C-labeled 127 amino acid casein substrates were indistinguishable (Supplemental Figure 6B). If there is any directional bias of Hsp104-catalyzed polypeptide translocation, it is not detectable in this experimental design.

**Polypeptide translocation mechanism of a potentiated Hsp104 variant**

Hsp104\textsuperscript{A503S} is a variant of Hsp104 engineered to provide enhanced disaggregation, specifically against aggregation-prone proteins implicated in neurodegenerative disease (7, 50). Remarkably, in addition to promising results in many \textit{in vitro} and yeast studies, this potentiated variant prevents neurodegeneration in a \textit{C. elegans} model of Parkinson’s disease (7). Moreover, Hsp104\textsuperscript{A503S} rescues FUS aggregation in mammalian cells (8). Could a difference in processivity relative to WT Hsp104 be the basis for the therapeutic gain of function exhibited by Hsp104\textsuperscript{A503S}? To test this possibility, we repeated the experiments schematized in Figure 1B with the Hsp104\textsuperscript{A503S} variant in place of Hsp104\textsuperscript{WT}.

Figure 2D (solid traces) displays representative time courses from the Hsp104\textsuperscript{A503S}-peptide complex prepared with RepA\textsubscript{Flu1-30}, RepA\textsubscript{Flu1-40}, and RepA\textsubscript{Flu1-50}. Comparing the time courses from Hsp104 and Hsp104\textsuperscript{A503S}, two differences are observed. First, the Hsp104\textsuperscript{A503S} time courses needed to be longer to capture the full reaction (compare insets in Figure 2A&D). Hsp104\textsuperscript{A503S} is in contact longer with the peptide compared to Hsp104. Second, in contrast to the time courses in Figure 2A for Hsp104\textsuperscript{WT}, a lag is not obvious in the Hsp104\textsuperscript{A503S} time courses (Figure 2D). Nonetheless, the Hsp104\textsuperscript{A503S} time courses were fit in the same manner as those from WT protein to test for the presence of a lag not detected by visual inspection and to make direct comparisons of the parameters between the time courses generated by Hsp104 and Hsp104\textsuperscript{A503S}. The time courses were individually fit to the equation derived from Scheme 2 as described in Methods. Best fit lines are shown as broken black traces in Figure 2D and parameters are summarized in the last three rows of Table 2 (means ± s.e., 3 replicates).

Consistent with our qualitative assessment, the quantitative analysis does not reveal a lag at any length of peptide tested (Figure 2D). Across the three lengths tested, \( n = (1.1 ± 0.1) \) steps (Figure 2E). Thus, at saturating ATP, the number of repeating steps is within error of unity. No additional repeats of the step with rate constant \( k_t \) were observed regardless of the length of the polypeptide substrate. These findings suggest that Hsp104\textsuperscript{A503S} therapeutic gain of function is not due to increased processivity against soluble polypeptides.

**Proposal of a minimal model for Hsp104-catalyzed polypeptide translocation**

The time courses presented in Figure 2 were collected in saturating ATP conditions (5 mM reaction concentration). Under these conditions, we observed that the kinetic step \( k_t \) was repeated one to two times with \( <n> = (1.6 ± 0.1) \) steps. If those steps represent translocation, then the observed repeating step would be coupled to ATP binding. This hypothesis predicts that the observed rate constant would depend on [ATP]. To test this possibility, we performed the experiments schematized in Figure 1B with varied concentrations of ATP in the second syringe.
Figure 3A-B shows a representative set of time courses from the Hsp104-catalyzed translocation of RepA_Flu1-50 with ATP concentrations ranging from 10 µM ATP (red trace) through 5 mM ATP (purple trace). Residuals from the fits of these time courses are shown in the supplemental information (see Supplemental Figure 7). The time courses are dependent upon ATP concentration, consistent with the idea that the signal is sensitive to ATP-driven steps that must occur before Hsp104 dissociates from the polypeptide. Inspection of the time courses reveals that the curves shift to the right, or are slower, with decreased ATP concentrations.

Consistent with our observations in the presence of saturating ATP (Figure 2A), the time courses in Figure 3A exhibit biphasic character across the full range of ATP concentrations tested. Likewise, a lag is observed in the time courses shown in Fig. 3A.

We sought to understand the functional dependence on ATP to describe the mechanism by which Hsp104 translocates polypeptide substrate. We demonstrated that the time courses collected as a function of substrate length (Figure 2A) could be described by Scheme 2, written generally for \( n \) steps. From analysis of those fits, we observed that \( n \) is between one and two steps. Scheme 3 represents a reduction of Scheme 2 and, in addition to \( k_{np} \) which was described in the context of Scheme 2, includes two observed rate constants, \( k_{obs,1} \) and \( k_{obs,2} \). Descriptions of the other variables are as defined for Scheme 2.

Each time course shown in Figure 3A&B was individually fit to Scheme 3 as described in Methods, with the fits shown in black broken lines. Parameters are displayed in Figure 3C-F (mean ± s.e., 3 replicates). It is important to note that the equation describing these time courses is symmetric. There is no information in the name of the parameter about the order in which each kinetic step occurs. The kinetic step with the rate constant reported as \( k_{obs,1} \) may occur before or after the kinetic step with rate constant \( k_{obs,2} \), and vice versa.

From the secondary plot in Figure 3C, \( k_{np} \) appears to be independent of ATP concentration with \( k_{np} = (0.011 ± 0.002) \text{ s}^{-1} \). The fraction of starting complex that begins in the productive complex, \( x \), varies across the range of [ATP] tested from a low of 0.53 ± 0.07 to a high of 0.78 ± 0.04 (Figure 3D). The higher \( x \) values are observed at higher [ATP], but many of the individual measurements are within error of each other such that a clear trend is not defined.

There is a clear dependence of \( k_{obs,1} \) on ATP concentration (Fig. 3E). This dependence is well described by a rectangular hyperbola given by

\[
k_{obs,x} = \frac{k_{obs,x,max} \cdot [ATP]}{K_{1/2} + [ATP]}
\]

From fitting the data in Figure 3E to Equation (3), we find \( k_{obs,x,max} = (0.56 ± 0.01) \text{ s}^{-1} \) and \( K_{1/2} = (1.3 ± 0.1) \text{ mM} \) (errors reported from the fit to Eq 3). A kinetic step that is in rapid equilibrium with ATP binding gives rise to hyperbolic dependence on [ATP] as observed in Figure 3E,
indicating that $k_{obs,1}$ is kinetically coupled to ATP binding. This indicates that at least one observed step is coupled to ATP binding and therefore represents an ATP driven step. We also note that this reported maximum rate constant is similar to previously reported Hsp104 ATPase rates from steady state experiments of 0.2-0.8 s$^{-1}$, although there are differences in the experimental conditions in the present study and these previous reports (34, 51, 52). In contrast, the rate constant $k_{obs,2}$ appears to be independent of ATP concentration across the range of ATP concentrations tested (Figure 3F). All of the points are within one standard error of the mean, $k_{obs,2} = (0.08 \pm 0.02)$ s$^{-1}$.

Earlier work has shown that ratios of ATP:ATPγS between 3:1 to 1:5 elicit enhanced disaggregase activity in the absence of Hsp70 (40, 53, 54). We did not observe such an effect in our experiments (see Supplemental Figure 8). The experiments that fall within this range of ATP:ATPγS ratios include the 30 µM through 300 µM ATP conditions, shown in the orange through green traces and marked with an asterisk in the in-plot legend in Figure 3 A and B. The region of the secondary plots that fall within this condition is also shaded in gray in Figure 3 C-F. One important difference between the experimental design used here compared to the activity assays used previously (40, 53, 54) is that our assay monitors a single turnover of the Hsp104-peptide complex. In contrast, prior experiments are performed over longer time frames, and allow for multiple rounds of Hsp104 engagement with polypeptide substrate. In addition, our assay uses a relatively short, model, unfolded peptide. This substrate is appropriate for a study of the molecular mechanism of polypeptide translocation. The substrates used previously include RepA dimers, aggregated GFP, and RepA1-70GFP in which the GFP portion is folded and fluorescent. Using differing experimental designs provides insight into different aspects of the mechanism.

$Hsp104^{A503S}$ translocation exhibits altered ATP coupling
The A503S mutation is located in the middle domain of Hsp104, a region implicated in modulation of Hsp104 ATPase activity (3). We tested whether the A503S mutation changes the way in which Hsp104 couples ATP binding and hydrolysis to polypeptide translocation. Thus, ATP concentration dependence experiments described for Hsp104$^{WT}$ in Fig. 3 were repeated with Hsp104$^{A503S}$.

Figure 4A&B show a representative set of time courses from the Hsp104$^{A503S}$ catalyzed translocation of RepA$^{Flu1-50}$ with ATP concentrations ranging from 10 µM ATP through 5 mM ATP. The time courses shown in Figure 4A&B are noted to be slower, or shifted to the right, as the ATP concentration is reduced. Again, this observation is consistent with the idea that the kinetic steps we detect in this assay are coupled to ATP binding and because the signal comes from the peptide these steps must occur before Hsp104 dissociates. At each [ATP] tested, the time course from Hsp104$^{A503S}$ (Figure 4A&B) is slower than the corresponding [ATP] time course from Hsp104 (Figure 3A&B). Another potentiated variant that exhibits similar activity to Hsp104$^{A503S}$, Hsp104$^{A503V}$ (7), displays increased disaggregase activity at ratios of ATP:ATPγS of 1:0 to 1:0.667 in the absence of Hsp70 (54). This range of ATP:ATPγS includes the 100 µM ATP and higher ATP concentrations shown as the light green through purple traces and marked with an asterisk in the in-plot legend in Figure 4 A and B.
Unlike Hsp104, no lag was observed in the data collected at saturating ATP for Hsp104\textsuperscript{A503S} (Fig. 2D). Here, we observe no lag across the range of [ATP] tested. Furthermore, Hsp104\textsuperscript{A503S} time courses were as well described by a sum of two exponentials as by the equation derived from Scheme 2 with $n = 1$. When the Hsp104\textsuperscript{A503S} time courses at saturating ATP (Figure 2D) were fit to the sum of two exponentials, one observed rate constant had the same value as the best fit parameter $k_{np}$ from Scheme 2 and the other observed rate constant had the same value as the best fit parameter $k_t$ from Scheme 2. The time courses generated by Hsp104\textsuperscript{A503S} do not exhibit a third kinetic step as observed for Hsp104\textsuperscript{WT}. Thus, these time courses are well described by a further simplification of Scheme 2, shown as Scheme 4 below.

$$C^* \xrightarrow{k_{np}} C \xrightarrow{k_{obr,1}} P$$

Scheme 4

In other words, one rate constant observed for the Hsp104\textsuperscript{WT} time courses is not observed in the Hsp104\textsuperscript{A503S} time courses. Two possibilities could explain this difference. One is that the kinetic mechanism is completely different for the Hsp104\textsuperscript{WT} versus Hsp104\textsuperscript{A503S} and so the kinetic steps observed for Hsp104\textsuperscript{A503S} are not those observed for Hsp104\textsuperscript{WT} translocation. The other possibility is that the point mutation (A503S) affected one or more of the steps observed in the WT mechanism, for example slowing a rate constant and/or affecting coupling to ATP.

Thus, for fitting the time courses shown in Fig. 4, we used an equation derived from Scheme 4. The low [ATP] time courses (10 $\mu$M, 30 $\mu$M, and 70 $\mu$M ATP) were well described by a single kinetic phase, consistent with the rate constant $k_{np}$. (54) The best fit lines are shown as broken black traces in Figure 4 A&B. Rate constants are shown in Figure 4C,E (means ± s.e., 3 replicates).

Consider the observed rate constant $k_{np, A503S}$ (Figure 4C, red circles). This rate constant $k_{np, A503S} = (0.0056 \pm 0.0007) \text{s}^{-1}$ is independent of ATP concentration and is the slower of the two observed rate constants. In our examination of Hsp104\textsuperscript{WT} time courses, $k_{np, WT}$ was independent of [ATP] and was the slowest observed rate constant at $k_{np, WT} = (0.011 \pm 0.002) \text{s}^{-1}$. If the rate constant $k_{np, A503S}$ reflects the same step as $k_{np, WT}$ (Figure 4C, black squares), then this kinetic step is twice as fast for Hsp104\textsuperscript{WT} as for Hsp104\textsuperscript{A503S}. Note that the value of $x$, the fraction of Hsp104-peptide complex in the productive form C, remains relatively flat at ~0.4 across all ATP concentrations tested for Hsp104\textsuperscript{A503S}, while the value of $x$ for WT increases slightly with increasing ATP (Figure 4D). This suggests that A503S binds ATP\textgamma{}S more tightly than does WT. For WT, higher ratios of ATP\textgamma{}S:ATP result in similarly low values of $x$ (Supplementary Table 3, second row). The observation that the A503S mutation alters nucleotide binding and hydrolysis is consistent with the mutation’s location within the regulatory M domain.

Finally, we observe a clear dependence of $k_{obr,1, A503S}$ on ATP concentration, Figure 4E (red circles). These data were well described by a rectangular hyperbola (Figure 4E, red line), Equation (3). From fitting the data in Figure 4E to Equation (3), we find that $K_{1/2, A503S} = (160 \pm 40) \mu$M and $k_{obr,1 \ max, A503S} = (0.100 \pm 0.005) \text{s}^{-1}$ (errors reported from the fit to Eq 3 ). This rate constant could reflect the same kinetic step observed for the WT protein as either $k_{obr,1, WT}$ (Figure 4E, blue circles) or $k_{obr,2, WT}$ (Figure 4E, black squares).
If we compare the ATP-dependent rate constant \( k_{\text{obs,1,A503S}} \) (Figure 4E, red circles) with the ATP-dependent rate constant \( k_{\text{obs,1,WT}} \) (Figure 4E, blue circles), we note that the maximum rate constant is 5-fold lower for A503S compared to WT, and that ATP affinity is an order of magnitude tighter for A503S than for WT. Alternatively, if we compare the ATP-dependent rate constant \( k_{\text{obs,1,A503S}} \) (Figure 4E, red circles) with the ATP-independent rate constant for Hsp104\(^\text{WT}\) (\( k_{\text{obs,2,WT}} \), Figure 4E, black squares), we note that the maximum rate for A503S is indistinguishable from the rate observed for WT. This finding would indicate that the apparent ATP-independence of \( k_{\text{obs,2}} \) for Hsp104\(^\text{WT}\), may be the plateau of a rectangular hyperbola reflecting tight ATP binding affinity. A rectangular hyperbola with a midpoint at 50 µM ATP is shown in black broken trace in Figure 4E as an example of how a high affinity isotherm for \( k_{\text{obs,2,WT}} \) as a function of [ATP] may look. In other words, the \( K_{1/2} \) of \( k_{\text{obs,2,WT}} \) for Hsp104\(^\text{WT}\) (Figure 3F) may be tighter than we can observe with this range of ATP concentrations. In that case, the A503S mutation has weakened the ATP affinity of this step compared to WT. Whether \( k_{\text{obs,1,A503S}} \) represents the same kinetic step observed as \( k_{\text{obs,1,WT}} \) or \( k_{\text{obs,2,WT}} \), this observation suggests that the A503S mutation impacts the coupling of ATP binding to the observed rate constant. The third possibility, that the step reflected in \( k_{\text{obs,1,A503S}} \) is a step not observed for the Hsp104\(^\text{WT}\), cannot be ruled out until the identity of these steps is determined.

**Discussion**

Engineered interactions between Hsp104 variants and the protease ClpP led to a prevailing model of highly processive translocation of polypeptide, or complete threading, by Hsp104 (20). Indeed, HAP-ClpP effectively degrades amyloid fibrils (21). However, that method is potentially influenced by ATP independent degradation and cannot be used to indicate processive translocation as we have shown for ClpB. Nevertheless, recent high-resolution structural studies were interpreted to indicate a repeated 2 amino acid step size coupled to ATP binding and hydrolysis (25). Here, we sought to test the structural model using transient state kinetic techniques and soluble polypeptides that do not require non-native interactions with a protease.

Here we report evidence of Hsp104 taking, at most, two ATP driven kinetic steps before dissociating from the polypeptide substrate. Based on static cryo-EM structures Gates et al recently proposed a physical translocation step size of 2 amino acids per step (25). If Hsp104 processively translocates a polypeptide chain using repeated steps with a step size of two amino acids, based on the potential binding sites illustrated in Figure 1, we would anticipate a minimum of 13 ATP driven steps before Hsp104 dissociates from the short polypeptides used here. Further, we would anticipate substantially more steps on the 102 and 127 amino acid α-casein substrates. The single turnover experiments performed here are sensitive to the slowest steps that must occur before dissociation from the polypeptide substrate. However, if the two observed kinetic steps do represent mechanical movement and the step-size is 2 amino acids per step then this would indicate that Hsp104 translocates only four amino acids before dissociation. Using Equation (2) with \( m = 2 \) amino acids and \( N = 4 \) amino acids, then the processivity \( Pr = 0.61 \). This finding suggests that, like ClpB (23), Hsp104 may use a “tug and release” mechanism to resolve protein aggregates (Figure 5). It is important to note that the tug and release
mechanism that we have proposed would be mechanistically identical to the entropic pulling mechanism proposed by De Los Rios et al. (55).

The Cryo-EM results reported by Gates et al clearly indicate that there are extended and closed conformations bound to α-casein (25). Assuming that the process of vitrification does not perturb the equilibrium, then the observed distribution of states represent the distribution of states present in the sample at thermodynamic equilibrium before vitrification. As with all thermodynamic measurements there is no information on the path. With that in mind, there is no information in the electron micrographs on the path by which the extended and closed conformations arrived at the extended and closed conformations. Although the extended and closed conformations have been interpreted to represent translocation intermediates, the path information is not present within the observations. Consequently, it remains unknown if the closed conformation extends or if the extended conformation closes. An alternative explanation is that the extended and closed conformations could represent different binding modes on the α-casein substrate used in those experiments. For example, the extended and closed conformations could be the consequence of the α-casein binding N-terminally vs. C-terminally. Regardless of the interpretation there is no experimental evidence reported that indicates the extended and closed conformations represent translocation intermediates.

There are notable differences between the kinetic time courses collected for ClpB versus Hsp104. In particular, the same experimental design used with ClpB did not result in the observation of lag kinetics (23). The lag indicates that there is more than one kinetic step with similar rate constants. Thus, for Hsp104 the lag indicates that the kinetic steps we detect have a similar rate constant whereas the two steps we detect for ClpB exhibit rate constants that are approximately an order of magnitude different (23). Regardless of these differences, for both ClpB and Hsp104 the two steps we detect are coupled to ATP binding and they must happen before the enzyme dissociates from the polypeptide chain. Thus, the detected kinetic steps are consistent with ATP-driven steps.

The observation that the kinetic steps detected here are coupled to ATP binding implies that we should detect the same step in an ATPase experiment. However, to fully understand the coupling of ATP binding and hydrolysis with peptide substrate translocation, there is an urgent need for a focused investigation of the dynamic distribution of oligomeric states of Hsp104, nucleotide-linked assembly, and the coupling of ATP hydrolysis to the various oligomeric states observed in solution (38). Without this context, individual ATPase measurements have limited applicability. We show in the present work that ATP binding is coupled to the observed kinetic steps preceding dissociation of hexameric Hsp104 from the polypeptide chain. But many additional futile sources of ATP hydrolysis remain possible. For example, Hsp104 contains two ATP binding and hydrolysis sites per monomer and therefore 12 ATP sites per hexameric ring. Thus there are significantly more ATPase sites than may be involved in the energy driven steps in a hexamer bound to a peptide substrate. There are even more sources of ATP hydrolysis possible if lower order oligomers hydrolyze ATP, which is currently unknown. A series of studies are underway to characterize the self-association of Hsp104 hexamers, the nucleotide-linked oligomerization of Hsp104, and the ATPase activity of Hsp104 in a dynamic equilibrium of oligomeric states.
The substrate itself may impact the translocation mechanism of Hsp104. For example, a more structured substrate such as folded GFP or an amyloid aggregate may exploit the plasticity of the Hsp104 mechanism (53) and elicit additional translocation steps. Additionally, collaborating chaperones, such as Hsp70 and Hsp40, may affect the mechanism of Hsp104. Hsp104 can dissolve amyloid independently from the Hsp70 system (53, 56, 57), but Hsp104 disaggregate activity against amyloid and disorded aggregates can be greatly stimulated by the Hsp70 system (2, 53, 58). Conditions have been found and widely used in vitro to examine peptide binding, substrate activated ATP hydrolysis, and the threading, unfolding or disaggregation of protein substrates by Hsp104 in the absence of the Hsp70 system (7, 20, 34, 40, 59-61). Our recent work with E. coli ClpB and DnaK, the bacterial homologues of Hsp104 and Hsp70, respectively, revealed that DnaK at sufficiently high concentrations to saturate the binding to ClpB triggers a release of peptide substrate from ClpB (62). Thus, under normal cellular conditions DnaK binding to ClpB may sequester ClpB in a low peptide affinity state, and upon cellular stress DnaK dissociates from ClpB to bind denatured clients, thereby unleashing ClpB in a high peptide affinity state to disentangle aggregates through a tug and release mechanism (62).

Hsp104A503S was developed to target substrates associated with neurodegenerative disease. This potentiated variant has been shown to suppress toxicity, solubilize aggregates, and restore proper cellular location in yeast and mammalian studies (7, 41, 50). Hsp104A503S has also been shown to protect neurons from degeneration in a C. elegans model of Parkinson’s disease (7). We considered whether this variant may gain its increased disaggregation activity by enhanced processivity. Subjecting the potentiated variant Hsp104A503S to the same experimental designs revealed that Hsp104A503S takes even fewer kinetic steps than Hsp104.

The location, A503, of the single misense mutation that imparts these enhanced activities suggests a regulatory effect. This position is in helix L3 of the middle domain of Hsp104. The middle domain regulates communication between NBD1 and NBD2, and also serves as the site of Hsp70 binding (63-67). How does a single missense mutation in this position enhance Hsp104 disaggregate activity? While this remains an open question, our observations suggest that one effect of the mutation is to alter the coupling of ATP hydrolysis to translocation.

The unleashed disaggregate activity of Hsp104A503S corresponds to slower dissociation from the peptide substrate compared to the unmodified protein (compare Figure 3A&B with Figure 4A&B). This observation is consistent with other reports in the field. A mixture of ATP with the slowly hydrolyzable analogue ATPγS can increase Hsp104 activities including substrate unfolding, disaggregation, and reactivation(40). The bacterial homologue ClpB was shown to have increased unfolding capability when the wild type protein was “doped” with hydrolysis deficient mutants(68). Taken together, these findings suggest that modulating ATP hydrolysis in such a way as to extend the amount of time during which Hsp104 is engaged with the peptide substrate, may be key in regulating Hsp104 activity. Analogous to how one might untangle a strand of lights or a delicate necklace, the disaggregating motors may exert some mechanical force on exposed loops or ends of an aggregate, possibly even moving back and forth rather than in a constant direction. This concept is similar to “entropic pulling” proposed by Goloubinoff to explain the mechanisms of Hsp70s. Essentially, the bound disaggregate prefers greater freedom
of movement, which is achieved by gaining distance from the surface of an aggregate. The conditions that slow dissociation from the peptide substrate or increase the residence time of the motor on the substrate protein, are shown to be more effective for Hsp104-catalyzed disaggregation.

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Author contributions
C.L.D. and A.L.L designed experiments. C.L.D. performed experiments and analyzed the data. N.W.S. performed selected anisotropy experiments; N.W.S. and A.L.L analyzed anisotropy data. M.J., J.L, K.M, E.S., and L.C. expressed and purified proteins. C.L.D. and A.L.L. wrote the manuscript with contributions from J.L, N. W. S., K.M, M.J., and J.S. All authors read and approved the final manuscript.


Figure Legends

Figure 1. Possible orientations and directionality of Hsp104 on peptide substrate. Hsp104 hexamers are depicted in blue from a side view. Peptides are depicted as linked black filled circles and the N- and C-termini are labeled. Green stars indicate a fluorescent dye covalently bound at either the N- or C-terminus of the peptide. (A) Identification of domain orientation in Hsp104 schematic. (B) Schematic for transient state, single turnover kinetic experiments. Hsp104, ATPγS, and fluorescently labeled peptide are incubated in one syringe, while ATP and α-casein are incubated in the other syringe of a stopped-flow fluorimeter at 25 °C. The contents of the two syringes are rapidly mixed. Fluorescence is excited at 494 nm and emission is observed at 515 nm and above. (C-H) Possible orientations of the Hsp104-peptide complex formed in the presence of ATPγS. Arrow indicates direction of Hsp104 movement with respect to the peptide. Green star indicates location of fluorescein dye on the peptide.

Figure 2. Substrate length independence. (A) Representative time courses from the rapid mixing of Hsp104 (2 µM), ATPγS (300 µM), and fluorescently labeled RepA truncation peptide (100 nM) against ATP (10 mM) and α-casein (20 µM). Colored traces are representative time courses using 30, 40, and 50 amino acid RepA truncation substrates labeled at the N-terminus. Inset shows full time course for inspection of plateau region. Best fits from NLLS analysis of each time course, individually, to equation derived from Scheme 2 are shown overlaid in black broken traces. Parameters are summarized in the first three rows of Table 2. (B) Number of repeating steps, n, as a function of substrate length. Average of three replicates of individual fits to Scheme 2 are shown. For all lengths, n = (1.6 ± 0.1) steps where the average is shown as a black line and one standard error in either direction is shown as a gray line. (C) Representative time courses of Hsp104 translocation and dissociation from N-terminally labeled 102 and 127 amino acid truncations of αS1-casein. Fluorescence is excited using polarized light at 494 nm and fluorescence anisotropy is observed at 515 nm and above using a T format polarization accessory. (D) Representative time courses from assay as explained in (A) except with the potentiated variant Hsp104A503S. (E) Number of repeating steps, n, as a function of substrate length for Hsp104A503S. Average of three replicates of individual fits to Scheme 2 are shown. For all lengths, n = (1.1 ± 0.1) steps where the average is shown as a black line and one standard error in either direction is shown as a gray line.

Figure 3. ATP dependence of Hsp104 catalyzed polypeptide translocation. Representative ATP dependent Hsp104-catalyzed polypeptide translocation time courses are presented to show the overall shape of the curve throughout the length of the time course (A) as well as the early part of the time course where the lag is present (B). The experimental data are shown as solid colored traces. Each color corresponds to a different ATP concentration, with 10 µM ATP shown in red. Intermediate concentrations of ATP go through orange, green, and blue, with the highest concentration, 5 mM ATP, shown in purple. The ATP concentrations that fall within the range of ATP:ATPγS that enhance Hsp104 activity are denoted by a gray asterisk. The broken black traces represent the best fit lines of each time course, individually, to the equation derived from Scheme 3 using NLLS analysis. (C-F) Secondary plots of the parameters from individual fits of time courses to Scheme 3 as a function of [ATP]. Parameters are (C) k_{up}, (D) x (the fraction of complex in the C or productive conformation), (E) k_{obs,1}, and (F) k_{obs,2}. (mean ± s.e., n=3).
Figure 4. ATP dependence of Hsp104<sup>A503S</sup> catalyzed polypeptide translocation. (A & B) Representative ATP dependent time courses are shown. The experimental data are shown as solid colored traces. Each color corresponds to a different ATP concentration, with 10 µM ATP shown in red following the same color pattern as in Figure 3. The ATP concentrations that fall within the range of ATP:ATP<sub>γ</sub>S that enhance Hsp104<sup>A503V</sup> activity are denoted by a gray asterisk. Two time scales are presented to show the overall shape of the curve throughout the length of the time course (A) as well as the early part of the time course where the lag is present at high ATP for the WT protein but not for the variant (B). The broken black traces represent the best fit lines of each time course, individually, to a single exponential for 10 µM, 30 µM, and 70 µM ATP and to Scheme 4 for the remaining [ATP]. (C, D, and E) Panels C through E are secondary plots of the parameters from those individual fits as a function of [ATP] (means ± s.e, 3 replicates) with Hsp104<sup>A503S</sup> translocation parameters shown in red circles. In panel C, k<sub>np,WT</sub> is overlaid for comparison in black squares. The shaded region of the plot represents the range of ATP concentrations which correspond to the ATP:ATP<sub>γ</sub>S ratios that elicit enhanced Hsp104<sup>A503V</sup> activity. In panel D, x<sub>C</sub>, the fraction of complex in the C or productive conformation, from WT is again overlaid in black squares. In panel E, the red, circles are from the fits of the Hsp104<sup>A503S</sup> time courses with 100 µM ATP – 5 mM ATP. The red curve represents the best fit of the data to a rectangular hyperbola with k<sub>obs,1,A503S,max</sub> = (0.1000 ± 0.0002) s<sup>-1</sup> and K<sub>1/2,A503S</sub> = (160 ± 40) µM. Errors reported are from the fit of the data displayed in (E) to Eq 3. In panel E, rate constants from fits of the translocation time courses catalyzed by Hsp104 are overlaid in blue circles (k<sub>obs,1,WT</sub>) and black squares (k<sub>obs,2,WT</sub>).
dissociation, ClpB can bind another exposed area of the aggregate, and repeat the process of entropic pulling, dissociation, and rebinding. This may allow portions of the aggregate to refold correctly. With the addition of the Hsp70 system, dysregulating mutations, or certain ratios of ATP:ATPγS, the aggregate can be fully rescued by ClpB. Panel C shows the representative time courses for Hsp104 (dark blue) in the same experimental design. A lag is observed, but there is no length dependence. Hsp104 also takes one or two kinetic steps, coupled to ATP binding, before dissociating from the substrate. The rate constants for these steps are similar, giving rise to the lag. The mechanistic interpretation for Hsp104 is the same as that for ClpB. Hsp104 binds a disordered aggregate, tugs on loose loops or ends via entropic pulling (possible space sampled shown by gray rings), dissociates, and can then rebind elsewhere on the aggregate to repeat this process. Also like ClpB, Hsp104 alone cannot fully rescue disordered aggregates. Panel D shows the representative time courses for Hsp104A503S (light blue). They are similar to those observed for Hsp104 in that there is a lag and there is no length dependence. The time courses are slower compared to Hsp104, however, indicating a longer time during which Hsp104A503S is bound to the substrate. The pictorial display of the mechanism indicates that this allows for a greater range of sampled spaces or more entropic pulling. While the basic process is the same (bind aggregate, loosen aggregate by entropic pulling, dissociate, and rebind), the longer residence time of Hsp104A503S on the aggregate allows it to more effectively loosen the aggregate, such that this potentiated variant is able to fully rescue the aggregated proteins without the Hsp70 system or mixtures of ATP and ATPγS.

Table 1. Polypeptide substrates used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Length (aa)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RepAFlu1-30</td>
<td>30</td>
<td>Flu-C MNQSFISDIL YADIESKAKE LTVNSNNTVQ</td>
</tr>
<tr>
<td>RepAFlu1-40</td>
<td>40</td>
<td>Flu-C MNQSFISDIL YADIESKAKE LTVNSNNTVQ PVALMRLGVF</td>
</tr>
<tr>
<td>RepAFlu1-50</td>
<td>50</td>
<td>Flu-C MNQSFISDIL YADIESKAKE LTVNSNNTVQ PVALMRLGVF VPKPSKSKGE</td>
</tr>
<tr>
<td>RepA1-30Flu</td>
<td>30</td>
<td>MNQSFISDIL YADIESKAKE LTVNSNNTVQ C-Flu</td>
</tr>
<tr>
<td>RepA1-40Flu</td>
<td>40</td>
<td>MNQSFISDIL YADIESKAKE LTVNSNNTVQ PVALMRLGVF C-Flu</td>
</tr>
<tr>
<td>RepA1-50Flu</td>
<td>50</td>
<td>MNQSFISDIL YADIESKAKE LTVNSNNTVQ PVALMRLGVF VPKPSKSKGE C-Flu</td>
</tr>
<tr>
<td>caseinFlu102</td>
<td>102</td>
<td>C-terminal 101 AA of αS1-casein with N-terminal cysteine</td>
</tr>
<tr>
<td>caseinFlu127</td>
<td>127</td>
<td>C-terminal 127 AA of αS1-casein with N-terminal cysteine</td>
</tr>
<tr>
<td>casein127Flu</td>
<td>127</td>
<td>C-terminal 127 AA of αS1-casein with C-terminal cysteine</td>
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</table>
Flu, fluorescein dye covalently attached to the cysteine residue
Table 2. Kinetic parameters determined from individual NLLS fitting of Hsp104 time courses to Equation from Scheme 2

<table>
<thead>
<tr>
<th>protein</th>
<th>peptide length (amino acids)</th>
<th>$k_{np}$ (s$^{-1}$)</th>
<th>$x$</th>
<th>$k_i$ (s$^{-1}$)</th>
<th>n (steps)</th>
<th>variance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp104</td>
<td>30</td>
<td>0.032 ± 0.001</td>
<td>0.67 ± 0.01</td>
<td>0.133 ± 0.007</td>
<td>1.51 ± 0.09</td>
<td>1.95x10$^{-5}$ - 8.59x10$^{-5}$</td>
</tr>
<tr>
<td>Hsp104</td>
<td>40</td>
<td>0.010 ± 0.001</td>
<td>0.70 ± 0.04</td>
<td>0.106 ± 0.007</td>
<td>1.52 ± 0.08</td>
<td>3.45x10$^{-6}$ - 7.04x10$^{-6}$</td>
</tr>
<tr>
<td>Hsp104</td>
<td>50</td>
<td>0.0069 ± 0.0002</td>
<td>0.61 ± 0.01</td>
<td>0.147 ± 0.007</td>
<td>1.88 ± 0.02</td>
<td>2.89x10$^{-6}$ - 1.90x10$^{-5}$</td>
</tr>
<tr>
<td>Hsp104A503S</td>
<td>30</td>
<td>0.0085 ± 0.0009</td>
<td>0.44 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td>1.00 ± 0.04</td>
<td>1.62x10$^{-5}$ - 2.28x10$^{-5}$</td>
</tr>
<tr>
<td>Hsp104A503S</td>
<td>40</td>
<td>0.0053 ± 0.0002</td>
<td>0.36 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>1.22 ± 0.06</td>
<td>2.02x10$^{-6}$ - 1.04x10$^{-5}$</td>
</tr>
<tr>
<td>Hsp104A503S</td>
<td>50</td>
<td>0.0055 ± 0.0002</td>
<td>0.30 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>1.22 ± 0.06</td>
<td>3.57x10$^{-6}$ - 7.23x10$^{-6}$</td>
</tr>
</tbody>
</table>

* The variance column reports the range of values for the fits to Scheme 2 for three replicates.

The uncertainties on the parameters represent the standard error of three independent determinations.
Flu-peptide
A
NTD
NBD1 / M Domain
NBD2

Hsp104
ATPγS
protein trap

λex = 494 nm
λem = 515 nm +
A) ClpA

native protein

fully refolded protein

processive vectorial translocation

B) ClpB

disordered aggregate

partially refolded protein

ClpB dissociation, rebinding

C) Hsp104

Hsp104 dissociation, rebinding

partially refolded protein

D) Hsp104 A503S

Hsp104 A503S dissociation, rebinding

fully refolded protein