An Empirical Energy Landscape Reveals Mechanism of Proteasome in Polypeptide Translocation

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Abstract

The ring-like ATPase complexes in the AAA+ family perform diverse cellular functions that require coordination between the conformational transitions of their individual ATPase subunits^{1,2}. How the energy from ATP hydrolysis is captured to perform mechanical work by these coordinated movements is unknown. In this study, we developed a novel approach for delineating the nucleotide-dependent free-energy landscape (FEL) of the proteasome's heterohexameric ATPase complex based on complementary structural and kinetic measurements. We used the FEL to simulate the dynamics of the proteasome and quantitatively evaluated the predicted structural and kinetic properties. The FEL model predictions are consistent with a wide range of experimental observations in this and previous studies and suggested novel mechanistic features of the proteasomal ATPases. We find that the cooperative movements of the ATPase subunits result from the design of the ATPase hexamer entailing a unique free-energy minimum

for each nucleotide-binding status. ATP hydrolysis dictates the direction of substrate translocation by triggering an energy-dissipating conformational transition of the ATPase complex.

Introduction

The ring-shaped oligomeric ATPases control key biological processes including protein folding, transcription, DNA replication, cellular cargo transport and protein turnover¹⁻³. The 26S proteasome is an ATP-dependent protein degradation machine in the AAA+ (ATPases associated with diverse cellular activities) family of ATPases⁴. The proteasome holoenzyme consists of a barrel-shaped 20S core particle (CP) capped by 19S regulatory particles (RP) on one or both ends⁵. Each RP features a nine-member Lid subcomplex and a heterohexameric ring of AAA+ ATPases assembled from six distinct gene products (RPT1-RPT6) that share 85% sequence identity. These ATPases use the energy from ATP hydrolysis to mechanically unfold substrates and translocate them into the CP for proteolysis (Fig. 1A).

Processive translocation and degradation of protein substrates is critical for the biological functions of the ubiquitin-proteasome system^{4,6}. In previous investigations of the structural mechanism of proteasomal degradation, cryo-electron microscopy (cryo-EM) of the substrate-engaged proteasome captured seven states, E_{A1}-E_{D2}. E_{A1} and E_{A2} likely represent the resting states of proteasome; E_{D1} and E_{D2} are hypothesized to be involved in substrate translocation (Fig. 1-fig. supp. 1)^{7,8}. In these structures, an unfolded substrate primarily interacts with aromatic residues on the pore-1 loop (PL1) of each ATPase. These short structured loops form a right-handed helical “staircase” delineating the interior of the translocation channel. Changes in bound nucleotides at the ATPase interfaces are associated with rearrangements of the

architecture of the PL1 staircase and the proteasome-substrate interaction, as a result of both translational and pivot movements of these ATPases (Fig. 1B and fig. 1-fig. supp. 1)^{7,8}. One or two PL1s are disengaged from substrate interaction in each state. These disengaged PL1s usually occupy distal, or top, positions in the staircase away from the CP (Fig. 1A). To account for substrate translocation, we and others have proposed that when certain PL1s disengage and move to the top, substrate-engaged PL1s may move in the opposite direction, towards the CP. This conformational rearrangement may provide the power stroke to promote axial translocation of substrate (Fig. 1B)^{7,8}.

In spite of these structural insights, important questions regarding the dynamics of proteasomal structures remain, in particular how the six proteasomal ATPases coordinate during conformational transitions to achieve substrate translocation. Based on these cryo-EM structures, we previously proposed that the conformation and nucleotide-binding status of the ATPase hexamer may cycle consecutively through six different states with rotational equivalence, thus driving processive substrate translocation^{7,9}. A similar model of sequential transitions was proposed in an independent study of yeast proteasome structures⁸. Only two out of the six conformations proposed in the sequential-transition model were identified in these structural studies, and direct experimental study of the transition sequence has not been reported^{7,8}. Transiting into the next state involves multiple changes in nucleotide status and a complex conformational rearrangement of the ATPase hexamer. We still do not know the order of these events and how the chemical energy from ATP hydrolysis may be harvested to drive these transitions.

In addition, there are experimental findings that appear inconsistent with the predictions of a strict sequential-transition model. For example, mutations of the Walker-A (WA) or

Walker-B (WB) motifs on an ATPase impede its nucleotide-binding or hydrolysis activity, and are therefore predicted to inactivate the proteasome by blocking the transition sequence. However, Walker mutations on some ATPases of the proteasome are in fact well tolerated in yeast¹⁰⁻¹². Similarly, mutations of other functional motifs on different proteasomal ATPases have varying effects on protein degradation, leading to the hypothesis that the six ATPases may have nonequivalent roles in the proteasome activities despite their high levels of similarity in sequence and structure^{10,13,14}. These functional properties of the proteasome are not interpreted by the previous models and so far no alternative model that is consistent with both structural and functional observations has been suggested.

Computational approaches, such as molecular dynamics simulations, are frequently employed to decipher the structural dynamics of proteins¹⁵. However, despite recent advances it is still impractical to perform a full-atom simulation of a large system such as the proteasome at a biologically-relevant time scale (~100ms)¹⁶. We therefore developed a novel approach to simulate the conformational dynamics of the proteasomal ATPase hexamer by constructing a physical model based on the nucleotide-dependent free-energy landscape (FEL) of the ATPase complex. To obtain the FEL, we first performed comparative analysis on known proteasome structures to identify the primary degrees of freedom (DOF) of proteasome's conformational changes, and applied these DOFs as the conformational coordinates of the FEL. We then parameterized the free energy surface based on the mode of nucleotide-ATPase interactions, and experimentally determined the nine parameters of the FEL. Conformational changes of the ATPase complex are mapped to simple stochastic transitions on the FEL which evolves as the nucleotide status changes, driven by independent chemical processes at each nucleotide-binding pocket.

To address whether the simulated dynamics based on the FEL model recapitulates the actual dynamics of the ATPase complex, we experimentally tested the model predictions in a wide range of conditions that are distinct from the results used for model construction, as well as comparing the predictions with published results. We found that the FEL predictions were widely congruent with the experimental measurements in this and previous studies.

Our work introduces a new method for studying the dynamics of a complex protein machine such as the 26S proteasome, and provides a coherent explanation for a variety of structural and kinetic observations. It also provides a satisfying picture of the underlying mechanism by which the AAA+ hexamer on the proteasome operates in driving substrate translocation.

Results

I. Structure-based construction of the FEL of the proteasomal ATPase complex

In this work we elect a combination of the ATPase complex's conformation and the nucleotide distribution in the six ATPase pockets to specify a "state" of the proteasome, for consistency with nomenclature in structural studies. A description of the FEL of proteasome is represented by the potential of mean force of a specific proteasome population measured as a bivariate function of its conformational coordinates and the nucleotide distribution^{17,18}.

The conformational coordinates of the FEL are defined by the primary DOFs of proteasome's conformational changes, identified by comparing proteasome structures. We designate each conformation in the FEL by whether the interfaces of the six ATPase domains on

proteasome are open or closed, based on the observation that the solvent-excluded surface area (SESA) of these interfaces mostly adopts binary values in these cryo-EM structures (Fig. 1C and fig. 1-fig. supp. 2). A large SESA is associated with closed interfaces that appear to arrange the PL1s on neighboring ATPases into a staircase that interacts with substrate peptide. In contrast, a smaller SESA suggests an open interface and the relative geometry of the neighboring ATPases tends to vary (Fig. 1-fig. supp. 1). An ATPase subunit that is flanked by two open interfaces is disengaged from substrate interaction, as observed in the cryo-EM structures (Fig. 1-fig. supp. 1). In the FEL, we constrain the total number of open interfaces in each hexamer conformation to be either 2 or 3, as observed in the cryo-EM structures except for the E_A states which involve only one open interface (Fig. 1-fig. supp. 1) (see Discussion). This defines 30 conformations, after excluding 5 with ambiguity in assigning ATPase-substrate interaction due to symmetry (Supp. file 1; methods 9.2). States in the FEL are also differentiated by the nucleotide distribution in these ATPases. Each nucleotide pocket may be occupied by ATP, ADP, ATP- γ S (a slowly-hydrolyzing ATP analog) or no nucleotide. We ignore the transient ADP•Pi state due to the very weak affinity between free phosphate and the proteasome (Fig. 2-fig. supp. 1); also, no such state has been identified so far in proteasome structures. The total number of distinct states in the FEL model is therefore $30 \times 4^6 = 122,880$.

The molecular details of the nucleotide-ATPase interactions suggest a strategy to parameterize the free energy of each conformation as a function of its nucleotide status. Each nucleotide-ATPase interaction on the proteasome involves several conserved elements: the WA, WB, sensor I and sensor II motifs from the *cis* ATPase and the arginine fingers from the *trans* ATPase (Fig. 2A)¹⁹. The arginine fingers are the major components interacting with the γ - and β -phosphate groups on ATP at a closed interface. We found that the *cis* elements exhibited

rather minor rearrangements among different states of either substrate-free or substrate-engaged proteasomes (RMSD $\sim 0.58\text{\AA}$) (Fig. 2-fig. supp. 2). We therefore parameterized the total free energy of the ATPase hexamer as a sum of the contributions from each individual ATPase interface. Each interface's contribution is subdivided into three components: the basal energy E_b , which derives from direct interactions between adjacent ATPases at a closed interface; the pocket energy E_p from the nucleotide-*cis*-element interactions, which are similar for ADP and ATP; and the bridge energy E_{Br} , which differentiates ADP from ATP and originates from the engagement of arginine fingers and other *trans* elements with the γ - and β -phosphate on the nucleotide at a closed interface (Fig. 2B). The *cis* pockets of disengaged ATPases exhibit either low or no nucleotide density in cryo-EM maps, and are associated with slightly rearranged *cis* motifs ($p=0.014$) (Fig. 1-fig. supp. 1 and fig. 2-fig. supp. 2)⁷. We therefore assign a separate pocket energy E_p^{APO} for disengaged ATPases to reflect their low affinity for nucleotides. The chemical energy in ATP is excluded from the free energy calculation, since it does not explicitly contribute to the simulation of ATPase dynamics (see section II).

These energy parameters can in principle vary for the different ATPase subunits Rpt1~Rpt6. The magnitude of this variation is unclear. To reduce the model's complexity, we made an approximation that all six ATPases share an identical set of parameters. As described below, this approximation provides excellent agreement with kinetic measurements and is compatible with the asymmetric cryo-EM occupancies and effects of WB mutations. In section IV, we analyze the contribution of the proteasome Lid-ATPase interaction to the symmetry breaking among these ATPases.

II. Evaluating the FEL model parameters

We determined all nine parameters in the FEL model from the measurements in this study and previous work (Methods 9.3 & 9.4). A critical parameter is the difference in the bridge energies (E_{Br}) between ATP and ADP interfaces, which we find is key for determining the directionality of translocation. This parameter was measured using a single-molecule binding assay, based on the relationship between the dissociation constant (K_d) of the nucleotides and these energy terms. In the FEL framework, the nucleotide pockets are classified into three groups, according to how the K_d depends on the energy parameters (Fig. 3A). This designation varies with the status of the *cis* ATPase and the interface. We used a very low concentration (200nM) of Alexa647-conjugated ATP to limit the interaction to the strongest binding pockets (group 1 in Fig. 3A) on the human 26S proteasome which was immobilized on a passivated coverslip, and observed the interaction between ATP and the proteasome by TIRF (Total Internal Reflection Fluorescence) microscopy. The proteasome was fluorescently labeled on the 19S particle using a SNAP tag to allow accurate detection of colocalization with Alexa647-ATP. We varied the concentrations of competing unlabeled nucleotides (ATP, ADP, and ATP- γ S) in a steady-state measurement, to circumvent the possibility that conjugation with fluorophore may alter the nucleotide-ATPase affinity (Fig. 3B). ATP- γ S was employed to minimize nucleotide hydrolysis. This analysis yielded the following ratio of the inhibitor constant (K_i) for ATP : ADP : ATP- γ S = 1 : 7.9 (± 2.0) : 0.5 (± 0.15) (at 90% confidence interval), giving $e_{Br, ATP} - e_{Br, ADP}$ = -1.6(± 0.4) kcal/mol.

After determining the FEL parameters, we explored the basic features of the landscape. For the twelve ATPase-hexamer conformations that are characterized by either a single or two adjacent disengaged ATPase units, as in the observed E_D or E_C states, uniform ATP binding generates a flat FEL. The presence of ADP in a single pocket breaks the symmetry and lowers the relative free energy of a subset of conformations by 1.6 kcal/mol. The further addition of an empty pocket next to the ADP pocket produces a well-separated energy-minimum conformation in which the empty and ADP *cis* pockets are found in the disengaged ATPase and its counterclockwise neighbor (Fig. 2C). Although we did not impose any coupling between nucleotide status and ATPase conformation, this energy-minimum arrangement of nucleotide and conformation is identical to those observed in the E_{D1} and E_{D2} states of proteasome, possibility explaining the prevalence of this arrangement in proteasome structures as well as in related AAA+ ATPases (Fig. 1-fig. supp. 1)^{7,20,21}.

We simulated the dynamics of the ATPase complex as stochastic transitions in a discrete 12 (6 conformational + 6 nucleotide) dimensional space (Fig. 3-fig. supp. 1). The transitions between conformations were described as a simple bi-state process with a rate constant determined by the Arrhenius equation. The ATP cycle at each pocket proceeds independently, as described by the basic chemical rate equations. The off-rate of a nucleotide is calculated from its K_d as a function of the energy parameters, with the on-rate set at a constant (Fig. 3A; Methods 9.3 & 9.4). The detailed process of a conformational change is not considered explicitly (i.e. as “adiabatic”) in that the change of nucleotide distribution alters the FEL and causes repopulation of the conformational space of proteasome. We do not include any assumption on the coordination of the nucleotide cycles, or the coupling of the nucleotide and conformational changes, or any predetermined sequence of transitions.

To generate testable predictions from simulated ATPase dynamics, we introduced a substrate peptide that is mechanically coupled with the continuous segment of the PL1 staircase. In these simulations, the disengaged PL1s occupy the top staircase position, as observed in cryo-EM structures (Fig. 1B)^{7,8}. The unit step of translocation is defined by the axial separation between PL1s, corresponding to approximately two amino acids (AA) in the substrate polypeptide (Fig. 1B). The force coupling with a translocating substrate may affect the rates of ATPase conformational changes. We represent this effect as a titling of the FEL and simplify by ignoring the stochastic and sequence-dependent variations of these forces and introduced two constant values to capture the average effect on the forward and backward processes (Methods 9.4).

To experimentally determine these two force parameters and another parameter for defining the activation energy barrier, we employed a quantitative degradation assay based on the decay of a fluorescent reporter of the ubiquitylated cyclinB N-terminus fused with an infrared fluorescent protein iRFP (Fig. 3D)²². The N-terminal cyclinB is an unstructured protein that is efficiently degraded by the proteasome starting from its N-terminus^{23,24}. We find that degradation of cyclinB-iRFP follows Michaelis-Menten-like kinetics with $K_M = 5.6\text{nM}$ (Fig. 3E). We performed each measurement at substrate concentrations much higher than the K_M to maximize the signal-to-noise ratio, and tested five substrate concentrations, each with three replicas, to verify the saturation condition. It took an average of 55 seconds (i.e. the turnover time) for the proteasome to degrade a 46kDa cyclinB-iRFP molecule in a process that could be rate-limited by several steps including substrate commitment, unfolding and translocation (Fig. 3D)⁴.

To determine the actual rate-limiting step, we engineered a mutant of cyclinB containing only three lysine residues, and conjugated fluorescent ubiquitin chains onto these lysines²⁵. We examined this substrate using a single-molecule translocation assay developed previously (Fig. 3C)^{25,26}. The processive translocation of a substrate coincides with the stepwise removal of entire ubiquitin chains by the deubiquitinase Rpn11 on the proteasome, which was detected by TIRF to measure the ubiquitin copies on a substrate molecule (Fig. 3-fig. supp. 2). The decay rate of fluorescent ubiquitins on three-lysine cyclinB suggests a translocation rate of 10.5 ($\pm$ 0.8) AA/sec (Methods 7). A similar translocation rate was found in a measurement of $V_{\max}$ of proteasomal degradation²⁷.

With this translocation rate, a cyclinB-iRFP peptide containing 445 AAs would take at least 44 seconds to progress into the CP, i.e. 80% of the 55-second total turnover time. This suggests that translocation is the limiting step of the entire degradation process for this substrate. Degradation of these substrates is unlikely to be limited by deubiquitylation because substrates with multiple Ub chains are degraded faster than the same substrate with fewer chains²⁵. Therefore, in the experiments described below, we used the measured degradation rate of cyclinB-iRFP as an approximation for the translocation rate. This reporter assay also gives consistent results with direct single-molecule measurements under perturbed conditions, such as in the presence of ADP or ATP- γ S (Fig. 3C). We next determined the translocation rate of cyclinB-iRFP in the presence of 500 μ M ATP and different concentrations of ADP, and estimated the three translocation-related parameters using these data (Fig. 3F and methods 9.4).

III. The FEL-predicted kinetics of substrate degradation

Directly probing the dynamical behavior of the proteasomal ATPases is challenging. If the FEL model can recapitulate the actual ATPase dynamics, it may contribute valuable insights into the functional mechanism of the proteasome. To evaluate the consistency of the model with reality, we seek to examine the predictions of the simulated dynamics in experiments that are independent of those for model construction, as discussed below. The workflow and the main results are summarized in fig. 4-fig. supp. 1.

We found that the simulated ATPase dynamics at a steady state promotes a directional translocation of substrate into the CP, with variations due to the stochasticity of the dynamics (Fig. 4A). We first compared the simulated translocation rates of cyclinB-iRFP at different ATP concentrations with experimental measurements (Fig. 4B). Interestingly, the translocation-rate curve is non-monotonic and peaks around the physiological concentration of ATP. The FEL model accurately captures the quantitative features in both the up and down phases of the rate curve, each yielding a different insight. The EC_{50} value for ATP in the up phase is $45\mu\text{M}$, far from the K_d value of ATP or ADP at any binding pocket (Fig. 3A: $\sim 100\text{nM}$ for group 1, $\sim 3\mu\text{M}$ for group 2, $\sim 2\text{mM}$ for group 3). Guided by the FEL model, we found that the expression of this EC_{50} value is given by the ratio between the total rate of ATP hydrolysis and the on-rate of ATP (Methods 10).

The observed inhibition of translocation at high ATP concentrations (Fig. 4B) is likely due to the loss of ATPase cooperativity. This inhibition is not due to extra Mg^{2+} , proteasome disassembly, degradation-independent iRFP inactivation, or a general slowdown of ATP hydrolysis (Fig. 4-fig. supp. 2 to fig. supp. 5). As the FEL model suggests, coordinated movements of the ATPase subunits require the ATPase complex to have a unique energy-minimum conformation for each nucleotide status typically occurring during physiological

operation, so that, when the nucleotide status changes, these ATPase's conformations undergo a well-defined collective change, leading to translocation. Very high ATP concentrations bias the proteasome towards all-ATP status and a flat FEL, resulting in a loss of ATPase cooperativity and abrogating the directionality of translocation (Fig. 2C, 4A). This result is in contrast to the predictions of a sequential-transition model, which would predict a monotonic increase of translocation rate at increasing ATP concentrations, regardless of the parameters and reaction details, inconsistent with the observation (Methods 11).

The FEL-predicted translocation kinetics are also consistent with the results of competition experiments involving ATP- γ S. In the simulation (Fig. 4A), ATP- γ S introduces pauses between processive phases of substrate translocation, longer at higher concentrations, which closely resembles the ATP- γ S-induced translocation pauses in single-molecule force measurements of ClpXP, a proteasome-like ATPase in prokaryotes²⁸. In the reporter experiment, 36 μ M ATP- γ S inhibited the translocation rate by 50% in the presence of 500 μ M ATP (Fig. 4C), despite the fact that the difference in the apparent K_i values between ATP and ATP- γ S is only 2-fold (Fig. 3B). ATP- γ S alters the ATPase dynamics which in turn affects the kinetics of nucleotide turnover. This process is required to interpret the low IC₅₀ for ATP- γ S, as suggested by the FEL model.

We also performed an ATP- γ S competition experiment at a level where the high-ATP-inhibition effect is apparent (15mM ATP). Despite an overall reduction in translocation and a dramatic shift in IC₅₀, the FEL-predicted rates still closely match the experimental results (Fig. 4D). The FEL model also predicts that the translocation rate should linearly depend on the rate of ATP hydrolysis at varying ATP- γ S concentrations (Fig. 4C), consistent with a previous observation²⁷.

For structurally-stable substrates, unfolding may be a limiting step in proteasomal degradation. To test the FEL model in the context of such substrates, we created a fluorescent reporter by inserting a dihydrofolate reductase(DHFR) domain from *E. coli* between cyclinB and iRFP. We found that adding a DHFR ligand, folic acid, led to a dose-dependent stabilization of the ubiquitylated cyclinB-DHFR-iRFP in the presence of the proteasome, while the degradation of the original cyclinB-iRFP was unaffected (Fig. 5-fig. supp. 1). In the presence of folic acid, cyclinB-DHFR-iRFP degradation is still complete, or processive (Fig. 5-fig. supp. 2). The IC₅₀ value for folic acid in inhibiting the degradation is 800μM, much higher than the 1μM dissociation constant between DHFR and folic acid²⁹. This is likely because unfolding of the DHFR domain by the ATPase's actions primarily occurs when DHFR is transiently unliganded, consistent with the linear relationship between folic acid concentration and the inverse of the degradation rate (Fig. 5-fig. supp. 3 and methods 12). The FEL model suggests that folic acid lowers the overall efficiency of ATP utilization in degrading DHFR-containing substrates as reported previously (Fig. 5A)²⁷. We tested the degradation rates of ubiquitylated cyclinB-DHFR-iRFP with 800μM folic acid in an ATP-titration experiment, and found that folic acid did not affect the EC₅₀ value of ATP in the up-phase of the rate curve, although it lowered the peak degradation rate. However, folic acid significantly reduced the degree of degradation inhibition at high ATP concentrations (Fig. 5A, B). This is likely because the ATPase activity unfolding the substrate is less affected by high ATP concentrations than is translocation (Fig. 4-fig. supp. 5 and fig. 5-fig. supp. 4). To predict the degradation rate of cyclinB-DHFR-iRFP, we introduced one additional parameter to describe the unfolding rate of unliganded DHFR by proteasome in the FEL model. These qualitative features of the predicted degradation-rate curve in the ATP-titration experiment are insensitive to the choice of this parameter (Methods 12).

Molecular machines may occasionally run backwards due to the stochasticity of single-molecular dynamics. Simulation by the FEL model suggests that substrates with an unfolding-resistant domain would not stably engage with the proteasome but would instead escape at a rate determined by the backward kinetics (Fig. 5C). A stable ligand, such as methotrexate, can inhibit the degradation of cyclinB-DHFR-iRFP at a low concentration by preventing the unfolding of DHFR, resulting in partial cleavage of the substrate (Fig. 5-fig. supp. 1)³⁰. In a competition assay, degradation of the reporter cyclinB-iRFP was reduced by a nonfluorescent competitor cyclinB-DHFR-iRFP^{dark} (Fig. 5D). This inhibition was exacerbated in the presence of methotrexate (Fig. 5E). We measured the turnover time of the stable DHFR substrate in this assay and found the value comparable with the model prediction which was calculated as a first-passage time on a 20~30AA peptide track measured from the PL1s to the proteolysis sites in the CP (Fig. 5F) (Methods 9.5 & 14). Adding ATP-γS or ADP in the simulation reduces the first-passage time and facilitates the escape of stable substrates (Fig. 5C). These results are consistent with the experimental values for the turnover time in the presence of ATP-γS or ADP (Fig. 5F).

In summary, we found that predictions from the FEL model closely matches new experimental observations at nucleotide concentrations ranging across three orders of magnitude, in both the forward and the backward processes. The overall consistency with experiments is not sensitive to parameter uncertainty at a typical value of ~30% (Fig. 5-fig. supp. 5).

IV. Organization of proteasomal conformations in dynamical space

We further explore the features of the simulated ATPase dynamics and compare the predictions with additional results to examine this FEL approach. This analysis also provides

insights into the mechanism of the proteasome and rationalizes the effects of ATPase mutations in previous studies.

Simulating the ATPase dynamics gives the steady-state distribution of the proteasome at each conformation and the frequency of every conformational transition. The FEL model is built on fundamental physical and chemical processes and does not *a priori* specify the occupancy of any conformation, the transition pathway between conformations, or how the ATPases cooperate. Interestingly, in the simulation we found that the ATPase conformations self-organize into a transition network, with the E_D-like and E_C-like conformations predominating, as they do in the cryo-EM analysis (Fig. 6A)⁷. Although the main transition pathway among E_Ds resembles the previous sequential model, there are several side transitions, or branches, that are key to understanding some experimental observations.

To simulate the effects of an ATPase mutation, we abolished the ATP-hydrolyzing activity of one ATPase, for example Rpt3, in the simulation, and then analyzed the global dynamics of the mutated proteasome³¹. ATP hydrolysis at the Rpt3-Rpt4 pocket is important for the transition from the E_{D1} to E_{D2} cryo-EM state; its inactivation mimics a WB mutation and strongly reduces the transition frequency from the E_{D1}- to the E_{D2}-equivalent conformation in a FEL simulation. This loss of “flow” was compensated by an increase in the transition to an E_C-like conformation, so that the overall translocation rate was only reduced by 11% (Fig. 6B and fig. 6-fig. supp. 1). Transition networks with an inactivated ATPase also show an expansion of the E_D-like conformations in which the ATPases at the -2 or -3 positions are flanked by open interfaces (0=mutant, negative=counterclockwise); this trend is consistent with and may explain a previous cryo-EM analysis on WB mutant proteasomes¹¹.

The order of the elementary steps of reactions and transitions is an important part of the ATPase mechanism and is challenging to observe directly. Different sequences of these steps have been hypothesized to drive the orderly conformational transitions of the proteasome and other AAA+ ATPases^{7,20,32,33}. Our simulation identifies the most-likely reaction sequence by enumerating all the possible sequences that can promote transitions between two states and calculating their probabilities. For the E_{D1}-to-E_{D2} transition, the most-likely reaction sequence begins with ATP hydrolysis at the Rpt3-Rpt4 pocket, followed by ATP binding to Rpt5 and a conformational rearrangement of the ATPase complex. This conformational change exchanges the ADP-bound Rpt3-Rpt4 interface for an ATP-bound Rpt5-Rpt1 interface and drives a unit-step translocation of two AAs by rearranging the PL1 staircase (Fig. 6-fig. supp. 2). In this process, 1.6kcal/mol energy is dissipated. This energy-dissipating step is important for establishing the directionality of translocation in the simulation. This conformational change also drives Rpt4 into a disengaged APO state with a weak nucleotide affinity, allowing rapid release of bound ADP. Otherwise, the rate of ADP release would be incompatible with the fast kinetics of substrate translocation. Phosphate release is unlikely to be limiting since phosphate has a K_i values of 70mM measured in a competition assay, a much weaker affinity than ADP for the ATPases (Fig. 2-fig. supp. 1).

Identifying this “most-likely” reaction sequence is critical for obtaining the formula for the EC₅₀ value of ATP (Methods 10). In a proteasome with non-hydrolyzing Rpt3, the E_{D1}-to-E_C transition that partially rescues the “flow” is initiated by ATP hydrolysis at the Rpt6-Rpt3 pocket. The associated conformational change drives a translocation of two unit-steps, or four AAs (Fig. 6-fig. supp. 3). These larger steps occur stochastically even in the wildtype proteasome, though they are less energetically favorable. Nonetheless, these occasional larger steps likely explain

the experimentally-observed translocation efficiency of 2.6~3.0 AAs per ATP consumption (Fig. 5A)²⁷.

We next address the question of whether our model can help to interpret the observed functional disparity among the proteasomal ATPases. The model described above is symmetric, with all ATPases having identical parameter. One potential source of symmetry-breaking is the proteasome Lid-ATPase interaction. The Lid subcomplex interacts extensively with the ATPase domains, primarily on Rpt3/6, in the E_A or E_A-like states in which the PL1s on Rpt3 and Rpt2 respectively occupy the top and bottom niches in the staircase and Rpt6 is in a disengaged position^{7,34-36}. This arrangement closely resembles one E_D-like conformation (Fig. 6-fig. supp. 4, grey node). Weakening the Lid-ATPase interaction by mutations reduces the fraction of proteasome in the E_A-like states³⁷. We therefore hypothesize that the Lid-ATPase interaction may effectively stabilize this specific ATPase conformation by lowering its standard free energy. We implemented such a stabilizing effect in the FEL simulation by lowering the free energy of this E_D-like conformation by an arbitrary value, and found that this resulted in significant expansion of the occupancy of the E_{D1}- and E_{D2}-conformations and a simultaneous shrinkage in other E_D-like conformations (Fig. 6-fig. supp. 4), consistent with the cryo-EM observations^{7,8}.

To study whether the Lid-ATPase interaction may contribute to the different growth phenotypes in yeast WB mutants, we examined how the translocation rate changed after simulated inactivation of the ATP-hydrolysis activity of individual ATPases in the presence of the Lid. We found that the predicted translocation rate is generally correlated with the corresponding growth phenotype in a range of the Lid-ATPase interaction strength, which suggests that this interaction may contribute to the growth phenotypes of yeast WB mutants, potentially in conjunction with other compensatory mechanisms (Fig. 6-fig. supp. 5).

Discussion

Protein machines accomplish complex tasks, driven by their exquisite structural dynamics at the molecular level. Studying these dynamics is challenging for both experimental investigation and molecular simulation.

In this work, we exercised the principle of parsimony to reconstruct the FEL of the proteasomal ATPase complex and experimentally determined its parameters in an attempt to uncover the mechanism in polypeptide translocation. The FEL is an intrinsic property of a protein or a complex and is a key component of physical models that have provided important insights into the mechanisms of molecular motors³⁸⁻⁴⁰. FELs can be derived from conformational occupancies in cryo-EM studies through the Boltzmann equation^{41,42}. Identification of the very large number of proteasomal states is challenging and the nonequilibrium transitions further complicate the analysis. The FEL-based simulation introduced in our study generates various predictions which we used to examine the validity of the simulated ATPase dynamics and this overall approach. We found that the simulation recapitulated a number of important experimental observations including degradation kinetics, state distributions in cryo-EM datasets and the growth phenotypes of WB mutants (Fig. 4-fig. supp. 1). Moreover, this study reveals that these varieties of phenomena are in fact driven by a coherent and simple principle embodied in our model, thus offering mechanistic insights into the ATP-driven cooperative actions of the ATPases in promoting the translocation of substrate polypeptides.

The observation that the nucleotide status of three consecutive ATPase subunits on the proteasome represents a continuous sequence in an ATP cycle has led to a sequential rotary model and “hand-over-hand” translocation for proteasomal ATPase activity^{2,7,8}. A strict sequential mechanism requires that the ATP-bound subunit adjacent to the ADP subunit must hydrolyze ATP first. We compared the nucleotide-interacting motifs in all high-resolution structures of the proteasome but failed to identify a consistent trend that could suggest an allosteric effect between the ATP-pocket and other parts of the complex. As an alternative, we considered the possibility that the apparent cooperativity between different subunits might emerge from the modulation of their collective FEL by ligand binding, as proposed in the celebrated Monod-Wyman-Changeux concerted model for allosteric regulation⁴³. Loss of ATPase cooperativity due to flattening of the FEL at above-physiological ATP concentrations reduces the rate of substrate degradation (Fig. 4B). A similar inhibitory effect has been reported for a bacterial pilus assembly motor, though the exact mechanism is still unclear⁴⁴. Although sacrificing some translocation efficiency, the flat FEL of the all-ATP state facilitates interchange among proteasomal conformations, even under physiological ATP concentrations. This property may be important for bypassing an occasional stuck or defective ATPase subunit that would completely inhibit proteasomal activity in a strict sequential-transition model. The resulting network of conformational transitions is analogous to the “ring-resetting” model for the ClpXP ATPase complex⁴⁵. It is interesting to note that the peptidase activity of the proteasome is also inhibited by ATP concentrations above a threshold; however, this is likely to be due to a different mechanism since the critical concentration for peptidase inhibition is ~50x lower than that for translocation⁴⁶.

One or two ATPase units are disengaged from substrate peptide or from the translocation channel in all identified states of the proteasome. These disengaged ATPases are flanked by two open interfaces except in E_A states, where the “disengaged” subunit is associated with one open interface. We speculate that this is due to the extensive Lid-ATPase interaction in these states³⁴. The presence of disengaged ATPases ensures that there is a unique energy-minimum conformation, and thus ensures a high level of cooperativity among the ATPase subunits. The smaller pocket energy E_p of these disengaged units also allows a fast nucleotide exchange, which is required for efficient translocation. Disengaged ATPase units are frequently observed in the structures of ring-shaped ATPases and may have a similar role in their activities^{20,47,48}.

The nonequilibrium transitions of the ATPase complex identified by the FEL approach reveal important features of proteasome functions. Our model shows that substrate translocation steps are directly coupled to an energy-dissipating conformational transition which swaps an ADP-bound closed interface with another ATP closed interface (Fig. 6-fig. supp. 2). We propose that this coupling may dictate the directionality of translocation. The simulated translocation process deviates from a strict “hand-over-hand” mechanism in that the step size in our model can adopt multiples of $2xAA$, depending on which ATP molecule is first hydrolyzed (Fig. 6-fig. supp. 2 and fig. supp. 3). Variations in step size have been observed in substrate translocation by ClpXP, though its connection with our simulation is unclear^{28,49}.

We used the cryo-EM structures of proteasome as the basis for model construction. These structures are mainly for identifying the DOFs of the ATPase’s conformational change (Fig. 4-fig. supp. 1). Other aspects of the structural information, such as the conformational occupancy and nucleotide distribution, are not required for model construction but are still consistent with its predictions. The inter-subunit signaling motif has been suggested to mediate

ATPase communication and coupling with nucleotides and may contribute to the bridge energy in the FEL model⁵⁰. We did not include explicit allosteric coupling between ATPases in our model, as we find that it is not essential for explaining the current experimental observations. Adding allosteric effects to the model would be straightforward and may accommodate the “coordinated bursts” mechanism to extend this model to other ATPase systems⁴⁷.

Partial degradation, or processing, by the proteasome is a natural process in the maturation of certain protein factors⁵¹. The backward process on proteasome is much less understood and may contribute to efficient release of partially-degraded substrates. In our study, modeling the substrate-escape kinetics as a first-passage time problem yields predictions consistent with the measurements. Other mechanisms, such as loss of grip on certain substrates, may also contribute to the escape kinetics⁵¹. In the current model, we simplified the mechanical force on a substrate during translocation as a constant. Incorporating the precise unfolding landscape of a substrate and the interaction energy with the ATPase into the FEL model would be straightforward⁵², and may shed light on the mechanism of partial or nonprocessive degradation of certain substrates.

The same mutation in the six highly-similar ATPases often have different effects on proteasome activities^{10,11,13}. Previous studies lead to no consensus on whether certain subunits play a more important role in substrate degradation and the extent to which the sequence divergence among the six ATPases contributes to these functional heterogeneities is unclear. Interestingly, we found that it is possible to rationalize at least some of these functional heterogeneities without invoking disparity at the level of individual ATPases. The introduction of Lid-ATPase interactions as a simple conformation-stabilizing parameter in the FEL simulation, recapitulates the asymmetric cryo-EM state distributions and may explain the different

phenotypes of WB mutants. Direct interpretation of WA mutants remains challenging, as these mutations often lead to proteasome assembly defects¹³. In the simulation, large heterogeneities of energy parameters among the six ATPases can alter the ATPase dynamics (Fig. 6-fig. supp. 6). Interestingly, this effect is significantly blunted by incorporating the Lid-ATPase interaction (Fig. 6-fig. supp. 6). This may contribute to the inessentiality of parameter variations in recapitulating the experimental results. Including the Lid-ATPase interaction in the model yields predictions that are compatible with all the kinetic measurements in our study and indicates that the proteasome may occasionally visit an E_A-like state during translocation. However, the peak translocation rate is underestimated by 20%-25% in this case, suggesting that our understanding of the symmetry-breaking mechanism is still incomplete.

The FEL presented here is undoubtedly an approximation of the actual FEL of the proteasome. Results in future studies may identify additional mechanisms, such as allosteric effects, to increase the accuracy of the simulated ATPase dynamics. As one example of the results that are not well recapitulated by the current model, protein substrates and ubiquitin chains activate the ATPase activity of the proteasome by ~2 fold, larger than the predicted 9% increase by the FEL model⁵³. This activation step may be independent of the processive translocation phase described by the FEL model, and may involve a transition from the resting to translocating states of the proteasome⁵⁴. In the E_A states of the proteasome, all ATPases bind nucleotides, suggesting that the disengaged subunit in the resting states may have higher nucleotide affinity than the disengaged ones in the translocating states⁷. This high affinity may limit nucleotide turnover in the resting states. Similarly, our model may not accurately describe the behaviors of substrates whose degradation is limited by steps other than translocation, such

519 as the DHFR-containing substrates in this study. Extending the FEL model to cover such
520 substrates may reveal other important aspects of the proteasome's activities.

521 We expect this FEL approach to have many applications in guiding experimental design
522 and data analysis, and to provide valuable insights into the mechanism of proteasomal ATPases
523 and other AAA machines.

524

525 **Methods and materials**

526

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Recombinant DNA reagent	pT7-CyclinB-iRFP (plasmid)	This study	pLM254	For expressing cyclinB-iRFP (available upon request)
Recombinant DNA reagent	pT7-cyclinB-DHFR-iRFP (plasmid)	This study	pLM428	For expressing cyclinB-DHFR-iRFP (available upon request)
Recombinant DNA reagent	pT7-cyclinB-iRFP-DHFR (plasmid)	This study	pLM429	For expressing cyclinB-iRFP-DHFR (available upon request)
Recombinant DNA reagent	pT7-cyclinB(K18, 36, 64) (plasmid)	This study	pLM120	For expressing cyclinB(K18,36,64) (available upon request)
Peptide, recombinant protein	Dylight550-Ubiquitin	Lu et al., <i>Science</i> (2015) ²⁵	Dy550-Ub	available upon request
Peptide, recombinant protein	Human 26S proteasome	HEK293 cell (Rpn11-HTBH)	hPTSM	
Peptide, recombinant protein	Human 26S proteasome SNAP-Rpn3	HE293 cell (SNAP-Rpn3) This study	hPTSM-SNAP	

Cell line (Human)	HEK293	Lab stock (commonly available)	HEK293	
Cell line (Human)	HEK293- SNAP-Rpn3	This study	HEK293- Rpn3-SNAP	for expressing SNAP-Rpn3 proteasome
Chemical compound, drug	ATP-gS	Sigma	A1388	
Chemical compound, drug	A647-ATP	Thermofisher	A22362	
Chemical compound, drug	Folic acid	Sigma	F8758	
Chemical compound, drug	Methotrexat e	Sigma	A6770	
Chemical compound, drug	Biliverdin	Sigma	30891	
Chemical compound, drug	SNAP- surface-549	NEB	S9112S	
Strain, strain background (E. Coli)	NiCo21 DE3	NEB	C2529H	
Software, algorithm	MATLAB 2018	mathworks		

Software, algorithm	Pajek	Pajek	http://vlado.fmf.uni-lj.si/pub/networks/pajek/	
Software , algorithm	Proteasome FEL model	This study	https://github.com/luyinghms/Proteasome-FEL-model.git	Source code for the FEL model
Chemical compound, drug	ATP-gS	Sigma	A1388	

527

1. Analysis of cryo-EM structures

The nucleotide-binding pockets were defined based on the structure of E_{A1} state. Briefly, for each Rpt subunit of E_{A1} state, the amino acids within 6~9 Å of the bound nucleotide were selected and defined as the nucleotide-binding pocket. Structures of each nucleotide-binding pocket in different states of the proteasome were aligned in Pymol and the RMSD of the aligned atoms in the nucleotide pocket was reported.

To gauge the interface between two ATPase subunits, the solvent-excluded surface area (SESA) was used to report the buried area formed by the interacting residues. Using Pymol, the solvent-accessible surface area (SASA) for individual ATPase domains in isolation and the SASA value for the complex were calculated. Then the SESA between two ATPase domains was calculated using this formula (using Rpt1 and Rpt2 as an example): SESA of Rpt1-Rpt2 = [(SASA of Rpt1) + (SASA of Rpt2) – (SASA of Rpt1-Rpt2 complex)]/2

2. Protein purification and labeling

Recombinant human N-terminal (1-88) cyclinB1 (cycB_NT), 3-lysine cycB_NT (K18, K36, K64), cycB_NT-iRFP, cycB_NT-DHFR-iRFP were purified from *E. coli* cells using a polyHis tag and Ni²⁺-affinity column. PKA (protein kinase A) sites (RRASV) was placed at both the N- and C-terminus of cycB_NT-iRFP and cycB_NT-DHFR-iRFP for detection by autoradiography in degradation assays.

Human ubiquitin with a cysteine residue and a polyHis-tag at the N-terminus was purified from *e. coli* cells using cation exchange chromatography(GE, 17-1152-01) and was labeled with Dylight-550-maleimide (Pierce, 62290). After removing unreacted dyes, labeled ubiquitin was subjected to anion exchange chromatography(GE,17-1153-01) to separate labeled and unlabeled ubiquitin. Finally, the N-terminal polyHis tag was cleaved off using thrombin.

Anti-20S antibody (MCP21) was biotinylated using biotin-NHS (Pierce, 20217), and was purified using a desalting column.

Radioactive ^{33}p -ATP was used to label substrates with a PKA site at the N-terminus for *in vitro* ubiquitylation and degradation assays.

Human E1, E2 UbcH10, wildtype(wt)-ubiquitin were purchased from BostonBiochem. Purified streptavidin was from Invitrogen.

Protein concentrations were determined using Bio-Rad protein assay; ubiquitin concentration was determined by UV A280 absorption.

3. Recombinant Anaphase-promoting complex/cyclosome (APC)-Cdh1 purification

Purification of recombinant APC-Cdh1 from insect cells has been described elsewhere^{55,56}. Briefly, viruses expressing 14 APC components were generated by transfecting Sf9 insect cells with the recombinant baculoviral genome based on a biGBac system using Fugene 6 reagent. Amplified viruses were added to HighFive insect cell culture for protein expression.

APC was expressed with a Twin-Step(II)-tag on the C-terminus of APC4, and was isolated from cell lysate using Strep-Tactin sepharose, and then was polished by ion exchange chromatography and gel filtration. Myc-6xHis-Cdh1 was purified from HighFive cells using Ni^{2+} agarose.

4. Affinity purification of the human 26S proteasome

Human proteasomes were affinity-purified on a large scale from a stable HEK293T cell line harboring HTBH tagged hRPN11(a gift from L.Huang). The cells were Dounce-homogenized in a lysis buffer (50mM NaH_2PO_4 [pH7.5], 100mM NaCl, 10% glycerol, 5mM MgCl_2 , 0.5% NP-40, 5mM ATP and 1mM DTT) containing protease inhibitors. The lysate was cleared and incubated with NeutrAvidin agarose beads (Thermo Scientific) overnight at 4°C. The beads were then washed with excess lysis buffer followed by a wash buffer (50mM Tris-HCl[pH7.5], 1mM MgCl_2 and 1mM ATP- Mg^{2+}). Usp14 on proteasome was washed off using the wash buffer plus 100mM NaCl for 30 minutes. 26S proteasomes were eluted from the beads by cleavage using TEV protease (Invitrogen).

For purifying SNAP-tagged proteasome, the HTBH-Rpn11 cell line was stably transfected with a lentivirus carrying FLAG-SNAP-Rpn3 under a CMV promoter, and the SNAP-proteasome was purified from the transfected cell line using the above procedure.

Identity of the cell lines is authenticated using STR profiling and mycoplasma contamination was regularly tested by PCR.

5. *in vitro* ubiquitylation reaction

The APC ubiquitylation reactions were carried out in the UBAB buffer (25mM Tris-HCL[pH 7.5], 50mM NaCl, 10mM MgCl₂) containing 30nM APC-Cdh1, 100nM E1, 2uM UbcH10, 2mg/ml BSA, the energy regenerating systems, 1uM substrate (cycB_NT, cycB_NT-iRFP, or cycB_NT-DHFR-iRFP), and 100uM wt-ubiquitin or 15uM Dylight550-ubiquitin, incubated for 4 hours at 25 degree. In case the substrates were ³³p-labeled, calyculin A(EMD, 19-139) was added at 10ug/ml to prevent dephosphorylation.

6. iRFP substrate degradation assay

Ubiquitylated iRFP substrates were diluted in a buffer containing 1xUBAB, 10% glycerol, 1mM DTT, 0.5ml/ml γ -globulin (Sigma G5009), 0.05% NP-40, and nucleotide-Mg²⁺ at the experimental concentrations, and were aliquoted to a 384-wells plate (Corning 3544). Purified human 26S proteasome was added at 1.0nM~3.0nM final concentration to start the reaction. Degradation kinetics was monitored using a microplate reader (BioTek Synergy H1) once every 90 seconds at 35 degree.

For each buffer condition, the degradation measurement was performed at five substrate concentrations (40nM, 60nM, 80nM, 120nM, 200nM), with three replicas for each concentration. A standard reaction with 500uM ATP-Mg²⁺ was included in every batch of reactions to control for sample batch-to-batch variations.

Data analysis

The initial degradation rate (v_0) was obtained using a linear fitting of the degradation curve in the first 15 minutes after temperature stabilization. The turnover time is the inverse of the degradation rate divided by the proteasome concentration.

7. Single-molecule proteasome translocation assay

The detailed procedure of single-molecule proteasome assay has been described before^{25,26}. Briefly, 15nM 26S proteasome and 15nM biotinylated MCP21 antibody were mixed and incubated at room temperature for 15 minutes. The proteasome-antibody mix was loaded

onto PEG-passivated slides coated with streptavidin. After a brief incubation, unbound protein was washed off, and was exchanged into an imaging buffer (1xUBAB, 20mM Imidazole, 2mg/ml BSA, nucleotides) containing diluted ubiquitylated substrate at ~1nM.

Images were acquired at 100ms per frame on a custom TIRF microscope equipped with three laser lines of 488nm(150mW), 561nm(150mW), 638nm(100mW), and a Pco sCMOS camera. The single-molecule experiment was performed at room temperature of ~27 degree.

Data analysis

The basic image processing and single-particle identification was performed as described previously^{25,26}. The “stepped” traces as in fig. 3-fig. supp. 2 which signaled the co-translocation deubiquitylation mediated by Rpn11 were identified and aligned by the time of substrate-proteasome interaction. The average intensity of fluorescent ubiquitins among these ‘stepped’ traces was calculated for each time point, and the rate of translocation was calculated using a linear fitting of the averaged trace between 1 to 3 seconds after proteasome-substrate interaction. Data analysis was carried out in Matlab 2018.

8. Single-molecule proteasome-nucleotide interaction assay

The coverslip surface was passivated with tween20 and functionalized with biotinylated BSA as described previously⁵⁷. 50nM SNAP-Rpn3 proteasome was incubated with 15nM biotinylated MCP21 antibody and 1uM SNAP-surface 549 dye for 30 minutes at room temperature. The proteasome-antibody mix was buffer-exchanged into buffer W (1xUBAB, 0.05% Tween20, 0.5mM DTT) + 0.4mg/ml BSA using a 30kD concentrator, and then was buffer-exchanged into buffer W + 1uM Alexa647-ATP at 4 degree. The sample was diluted by 5 times in an imaging buffer (buffer W + competitive nucleotides + 200nM Alexa647-ATP (Thermo Scientific) + PCA/PCD as the oxygen scavenging system) and was incubated for 20 minutes on ice before loading onto the passivated surface via streptavidin. Images acquisition started after a 3-minute incubation. We did not observe obvious 19S-20S dissociation as suggested by the fluorescent SNAP-Rpn3 signal in a 30-minute incubation.

Data analysis:

The basic image processing and single-particle identification was performed as described previously^{25,26}. We performed colocalization (<1 pixel) analysis of the SNAP-tag signal with

Alexa647-ATP. The fraction of SNAP-tagged proteasome particles that interacted with Alexa647-ATP under a steady-state condition was calculated as the colocalization ratio (C. R.). The inhibitor constant K_i value for each type of nucleotide was obtained by a linear regression using the following formula:

$$C. R. = \frac{[ATP^*]}{K_{ATP^*} + [ATP^*] + [NT]}$$

C.R.: colocalization ratio; [ATP*]: concentration of Alexa647-ATP; K_{ATP^*} : dissociation constant of Alexa647-ATP; [NT]: concentration of competing nucleotide. Data analysis was performed in Matlab 2018.

9. Constructing the nucleotide-dependent free-energy landscape (FEL) of the ATPase complex on proteasome to simulate its conformational dynamics

9.1. Basic definitions and assumptions.

- **Conformation:** A conformation here is narrowly defined as a geometric arrangement of the six ATPases, regardless of their bound nucleotides. Totally 30 conformations are included in the FEL (§9.2).
- **State:** Unless specified in the context, a state here refers to a unique combination of an ATPase conformation and a nucleotide distribution in the six binding pockets. Since each nucleotide pocket can be empty, ATP-bound, ADP-bound, or ATP-γS-bound, the total number of states in the nucleotide-dependent FEL is $30 \times 4^6 = 122880$.
- **Dynamical space:** a complete description of the conformational changes of the proteasomal ATPase complex, including the conformations, the conformational occupancies, the conformational transition rates and their dependence on the nucleotide distribution.

We assumed that the six ATPases on proteasome were associated with the same set of energy and kinetic parameters. The substrate peptide was assumed to be tightly gripped by the pore-1 loops (PL1) on the ATPases without slippage during translocation.

9.2. Defining a discrete conformational space of the proteasome ATPase complex by extrapolating the cryo-EM observations

We designated each conformation in the FEL by the states of its six ATPase domain interfaces, either open or closed. We constrained the total number of open interfaces in each conformation to be either 2 or 3, since only 2 or 3 open interfaces have been observed in the cryo-EM states of the proteasome. Five conformations having 3+3 or 2+2+2 symmetry in the ATPase architecture were excluded to avoid ambiguity in assigning substrate-PL1 interaction. After all, the FEL contains totally 30 discrete conformations (Supp. file 1). Four conformations have so far been identified by the cryo-EM structural analysis.

Based on the cryo-EM observation, we incorporated the following rules to simulate substrate translocation: a series of closed interfaces positioned the PL1s on consecutive ATPases into a staircase arrangement; the axial separation between adjacent PL1s was 2xAAAs. Open interfaces did not constrain the relative geometry between adjacent ATPases. If one or two consecutive ATPase subunits were flanked by open interfaces at both ends, they were disengaged from substrate interaction, and their PL1s moved to the top registry of the staircase (Note 1). Disengaged ATPases were assigned with a different set of energy parameters to reflect the *APO* configuration of their nucleotide *cis* pockets in cryo-EM structures. In the scenarios involving three ATPase segments, the largest segment was assumed to interact with the substrate. The distance between the 20S and the PL1 at the lowest staircase position was considered invariant. The distance of substrate translocation of each conformational change is taken as the difference between the average staircase positions of those PL1s that interact with the substrate both before and after the transition.

*Note 1: As long as these ATPases are disengaged from substrate interaction and do not block the movement of other ATPases, whether they stay at the top registry or not does not affect the simulation result.

9.3. Determining the FEL and kinetic parameters

1. $k_{on} = 1e5$ /M/sec for all the ATPase pockets and nucleotides. No measurement of the nucleotide k_{on} for proteasome has been reported in our record. To estimate the upper bound of k_{on} , we use the observation that ADP-filled interfaces in cryo-EM structures had approximately equal tendency to be open or closed, which requires $e_{Br,ADP} \sim 0$ ($\pm kT$) (Fig. 1C); this condition can

only be achieved if $k_{on} < 3e5$ /M/sec. To estimate the lower bound, we consider that the proteasome can still translocate and degrade substrates efficiently at $\leq 100\mu\text{M}$ ATP (Fig. 4B). This requires $k_{on} > 2e4$ /M/sec, otherwise nucleotide rebinding would consume too much time, inconsistent with the fast translocation kinetics. Therefore, we estimated k_{on} as the geometric mean of its upper and lower bounds. This on-rate estimation is consistent with that calculated directly from the ATP EC50 expression (Fig. 4B and methods 10). This value is also consistent with the nucleotide k_{on} for F1-ATPase, in spite of its divergence from proteasomal ATPases⁵⁸.

2. $e_p^{APO} = -3.7$ (kcal/mol) for ATP, ADP and ATP- γ S at an *APO* pocket. We noticed that the disengaged ATPases were nucleotide-bound in the yeast proteasome cryo-EM structures despite high similarity with the human proteasome conformations^{7,8}. This, we surmise, is because 5mM ATP was used in the yeast proteasome study while 1mM ATP was used in our human proteasome project. In addition, higher-than-2mM concentrations of ATP- γ S have been found to alter the proteasome conformational distribution in a bulk measurement, which should be due to the nucleotide interacting with a weak pocket that has a comparable K_d ¹¹. Therefore, we set the $K_d = 2\text{mM}$ for an *APO* pocket, which is equivalent to $e_p^{APO} = -3.7$ (kcal/mol).

3. $e_p = -7.4$ (kcal/mol) for ATP, ADP and ATP- γ S. Nucleotide affinity for the group-2 pockets should be independent of the arginine fingers which allows us to determine $K_d^{(2)}$ value in Fig. 3A from a published result. In a previous study on the PAN complex which is the proteasomal ATPase homologue in archaeobacteria and has a similar staircase architecture as the proteasome, the nucleotide off-rate was measured, and one time component (~ 3 sec) was not affected by the arginine finger mutation on the PAN complex, which should be associated with the group-2 pockets^{20,59}. The *APO* pocket parameters are also arginine-finger independent, however, its off-rate is about 100x higher, easily separable from this result. Therefore, this analysis identifies $K_d^{(2)} = 3.3\mu\text{M}$, which gives $e_p = -7.4$ (kcal/mol).

4. $e_{Br,ADP} = -0.52$ (kcal/mol) $e_{Br,ATP} = -2.1$ (kcal/mol). From the single-molecule nucleotide proteasome interaction measurement, we obtained $e_{Br,ATP} - e_{Br,ADP} = -1.58$ kcal/mol. In a study using rabbit 26S proteasome, ATP- γ S promotes the peptidase activity of proteasome likely by restructuring the ATPase complex⁴⁶. The EC₅₀ value of ATP- γ S in this assay should be mapped to the strong pockets or group-1 in Fig. 3A, which gives $e_{Br,ATP} + e_p = -9.5$ (kcal/mol) ➔

$e_{Br,ADP} = -0.52$ (kcal/mol) & $e_{Br,ATP} = -2.1$ (kcal/mol). ATP- γ S is assumed to have the same energy parameters as ATP.

5. $e_b = 0$ for all the interfaces. The basal energy represents in the nucleotide-independent affinity between ATPases. The RP tends to dissociate from the CP in the absence of nucleotide⁶⁰. The nucleotide-free RP structure suggests an open ring with broad ATPase interfaces, indicating the intrinsic affinity between ATPases is weak (~ 0 kT)²². When simulating the transitions, we assigned a small random value (~ 0.2 kcal/mol) for each interface to resolve the ambiguity due to energy level degeneracy. We randomized these values in each simulation.

6. k_{off} was determined for individual pocket according to the following equation.

$$k_{off} = k_{on} * e^{(E_p + E_{Br})/kT} * C_0 \quad (1)$$

where, E_p , E_{Br} is the pocket and bridge part of the free energy at a specific pocket. C_0 is the standard concentration (1M).

9.4. Determining the parameters related with substrate translocation

The transition rate between conformation A and B is generally described by the following Arrhenius equation:

$$r_{A \rightarrow B} = k_0 e^{(E_A - E_B - f_{AB} * d_{AB})/2kT} \quad (2)$$

Where E_A , E_B are the standard free energy of conformation A and B at a given nucleotide status; f_{AB} is the average force on substrate during the translocation; We assigned two separated values of f_{AB} for the forward and backward translocations to reflect the dissipative and conservative parts of this force; d_{AB} is the translocation distance associated with the A $\rightarrow$ B conformational change (§9.2). k_0 is a constant pertaining to the activation energy barrier. The factor '2' comes from detailed balance.

We determined the three parameters necessary for specifying the translocation kinetics: the average force exerted on the substrate peptide during forward (towards the CP) translocation; the average force during the backward translocation; the energy-barrier parameter k_0 . We varied the concentration of ADP-Mg²⁺ in the presence of 0.5mM ATP, and measured the degradation rate or the equivalent translocation rate of ubiquitylated cyclinB-iRFP by purified human 26S

proteasome as described in Fig. 3F. We scanned the relevant range of the three parameters and determined the translocation rate $\sim [\text{ADP}]$ at each parameter combination (§9.5). The best parameter trio matching the experimental observation at all ADP concentrations was chosen:

7 (index following §9.3). f_{AB} during forward translocation = 1.8pN

8. f_{AB} during backward translocation = 0.56pN

9. $k_0 = 72 \text{ sec}^{-1}$

10. $k_h = 1.4 \text{ ATP/sec/subunit}$, zero for ADP or ATP- γ S. k_h is the ATP hydrolysis rate of each pocket. The total rate of ATP hydrolysis was calculated in a simulation of translocation as described in §9.5. The hydrolysis rate was chosen to match the experimental values of 3.8~6 ATP/sec/19S²⁷. From current cryo-EM structures, we did not identify any evidence supporting allosteric effect to up- or down-regulate the ATP hydrolysis activity at certain nucleotide pockets in the ATPase staircase. We therefore assigned the same hydrolysis rate to all the pockets at closed interfaces, since Arginine fingers are required for ATP hydrolysis^{19,46,59}. We have tested alternative assumptions by restricting the hydrolysis activity to the subunits close to the 20S and found that the qualitative features of the simulation results remained. Nonetheless, the current k_h assignment provided the best overall quantitative consistency with the experimental data.

9.5. Monte Carlo simulation of the FEL model of proteasome

The starting condition was set to the E_{D1} state. The conformation of the ATPase hexamer evolved according to the FEL at the current nucleotide status and equation (2). The nucleotide statuses at the six pockets independently evolved according to the kinetic constants at each pocket. We did not impose additional constraints or allosteric coupling between nucleotide pockets or with ATPase conformations.

For each simulation, the system was evolved for 400,000 steps corresponding to ~1000sec in physical time that is sufficient to achieve a steady-state distribution. A block diagram for the simulation algorithm was included in Fig. 2-fig. supp. 2.

For simulating the backward process, the forward translocation was set up zero when the translocated AAs $\geq L_0$ which is the length of the peptide track. L_0 ranges from 20 to 30 AAs because of the uncertainty of defining the distance between PL1s to the proteolytic sites in the

CP. Therefore, when the translocated AAs = 0, we assume the substrate will escape from the proteasome. Substrate reentry is not considered due to a loss of the ubiquitin signal from the cyclinB segment.

For simulating a Walker-B motif mutant, the hydrolysis rate constant of the mutated subunit was set to zero.

For simulating Lid-ATPases interaction, the standard free energy of an E_D-like conformation which mimics the ATPase architecture in the E_A states was lowered by an arbitrary value. In this conformation, Rpt3's PL1 occupies the top position in the ATPase staircase while Rpt2 at the bottom and Rpt6 disengaged. We scanned this value from 1.6 to 4.0 kcal/mol for testing the consistency in each task.

9.6. Parameter sensitivity analysis

The values of the parameters are either decreased or increased by 30%, except for k_{on} which is varied by 3x due to the large uncertainty in its estimation. The translocation rates at all the nucleotide conditions in Fig. 4 are calculated using the FEL model and are compared with those at the original parameter values. The mean deviation of each parameter perturbation over the original translocation rate is calculated as the sensitivity score.

All the simulation was performed on the O2 cluster at Harvard Medical School using 48 cores, programmed in Matlab 2018 with the parallel computing toolbox. The source code is available from GitHub⁶¹ or by request. Network figures were generated using Pajek (<http://vlado.fmf.uni-lj.si/pub/networks/pajek/>)

10. Deriving a formula for the EC₅₀ value in the ATP titration experiment

The sequences of fundamental steps underlying a productive translocation was shown in fig. 5-fig. supp. 4. The total time for one translocation step is

$$t_{tr} = 1/k_h^{all} + \langle 1/r_{A \rightarrow B} \rangle + 1/(k_{on}[ATP]) + 1/k_{off}^{APO} \quad (3)$$

Where, k_h^{all} is the overall rate of ATP hydrolysis of the hexamer; “ $\langle 1/r_{A \rightarrow B} \rangle$ ” is the average conformational transition time.

Under limiting ATP concentration, $1/(k_{on}[ATP])$ becomes significant. Therefore

$$EC_{50}(ATP) = \frac{1}{k_{on}(1/k_{off}^{APO} + 1/k_h^{all} + \langle 1/r_{A \rightarrow B} \rangle)} \sim \frac{k_h^{all}}{k_{on}} \quad (4)$$

11. The translocation rate at different ATP concentrations as predicted by a strict sequential-transition model

The previously-proposed sequential model does not contain quantitative details^{7,8}. As a comparison with the FEL model, we adopt a simple form of sequential model without losing generality. This sequential model involves six equivalent kinetic segments in a cycle; each contains two processes: 1. binding/unbinding of ATP; 2. ATP hydrolysis and ADP release

If assuming functional symmetry of the six ATPases, the steady-state occupancy of $A_n = A_{n+1} \dots = A$ and $B_n = B_{n+1} \dots = B$, therefore under the steady-state condition:

$$0 = A * [ATP] * k_{on} - B * k_{off} - B * k_h$$

Considering $A + B = C^*$ is a constant due to the symmetry

821 The rate of translocation $\propto B \cdot k_h = \frac{[ATP] \cdot C^* \cdot k_{on} \cdot k_h}{k_{off} + k_h + k_{on} \cdot [ATP]}$, is a monotonic function of the ATP
 822 concentration.

823 **12. Interpreting the degradation kinetics of unfolding-limited substrates**

824 The total turnover time of a cyclinB-DHFR-iRFP molecule under a substrate-saturating
 825 condition involves the time for translocation and the time for unfolding.

$$826 \quad t_{total} = t_{tr}([ATP]) + t_{uf}([ATP]) \quad (5)$$

827 Unfolding of the DHFR domain mostly happens when DHFR is transiently unliganded. Because
 828 if that's the case, the inverse of the degradation rate of DHFR should linearly depend on the
 829 concentration of folic acid [FA] at any ATP concentration, according to equation (6). This is
 830 indeed supported by the observation described in fig. 5-fig. supp. 1.

$$831 \quad t_{total} = 1 / r_{deg} = 1 / r_{tr}([ATP]) + 1 / r_{uf} \left(1 + \frac{k_{on}^{FA} \cdot [FA]}{k_{off}^{FA}} \right) \quad (6)$$

832 where, r_{deg} is the degradation rate, r_{uf} is the unfolding rate of unliganded DHFR on the ATPase
 833 complex. k_{on}^{FA} , k_{off}^{FA} are the rate constants of folic acid on DHFR.

834 Substrate unfolding is likely achieved by the ATPase power strokes⁶². Although the exact
 835 mechanism is still poorly understood, the unfolding rate should be generally in proportion to the
 836 ATPase activity in the absence of unfolding intermediates⁶³. The overall degradation rate is
 837 predicted to be

$$838 \quad r_{deg} = \frac{r_{tr}([ATP])}{1 + \frac{r_{tr}([ATP])}{C_{uf} \cdot r_h([ATP])} \left(1 + \frac{k_{on}^{FA} \cdot [FA]}{k_{off}^{FA}} \right)} \quad (7)$$

839 One extra parameter C_{uf} was introduced as the unfolding rate coefficient in front of the ATPase
 840 activity r_h , which should generally depend on the folic acid concentration.

841 Translocation rate r_{tr} and ATPase activity r_h as functions of ATP concentrations were
 842 simulated using the FEL model. We adjusted C_{uf} to match the measured degradation rate of

cyclinB-DHFR-iRFP in the presence of folic acid (Fig. 4E). The qualitative features of the degradation-rate curve in Fig. 4F, i.e. nearly-identical with that of the FA-free curve in the up-phase and weaker effect of high-ATP inhibition, are independent of the choice of C_{uf} .

13. Measuring the ATPase activity of proteasome

100nM purified human 26S proteasome was incubated in a buffer (1xUBAB, 0.5mg/ml BSA, 0.05% NP-40, 0.5mM DTT) with varying concentrations of ATP-Mg²⁺ for 30 minutes at 30 degree. No-proteasome controls were incubated under an identical condition. 20uM denatured ovalbumin was added as a generic substrate of proteasome⁶⁴. 30nM and 50nM proteasome was used for 100uM and 300uM ATP respectively. After incubation, the concentration of free phosphate was quantified using Malachite green assay on a plate reader (BioTek Synergy H1).

14. Measuring the residence time of an unfolding-resistant substrate on the proteasome

An iRFP substrate degradation assay is set up as described (§6), involving 100nM polyubiquitylated cyclinB-iRFP reporter substrate and 50nM polyubiquitylated cyclinB-DHFR-iRFP^{dark} as the competitor, either in the presence or absence of 300nM methotrexate (MTX). cyclinB-DHFR-iRFP^{dark} is nonfluorescent due to the lack of biliverdin in iRFP. The residence time T_B , i.e. the mean time between when translocation reaches L_0 and escaping from the proteasome, of cyclinB-DHFR(MTX)-iRFP is calculated based on the formula:

$$T_B = \frac{(r_{RI0}/r_{RI} - 1)[R]}{[C]} T_R + \frac{r_{RI0}}{r_{RI}} \times T_{I0} - L_{IN}/v_t$$

r_{RI0} : reporter's degradation rate in the absence of MTX

r_{RI} : reporter's degradation rate in the presence of MTX

$[R],[C]$: concentrations of reporter and competitor

T_R : turnover time of the reporter (55 sec)

865 T_{I0} : turnover time of competitor in the absence of MTX (96 sec)

866 L_{IN} : length of the cyclinB: 90AA

867 v_t : translocation rate (10.5AA/sec)

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authors declare no conflict of interest. **Data and materials availability:** All data generated or analyzed during this study are included in the manuscript and supporting files. Source data files have been provided for Figures 3,4,5. The original images for the single-molecule experiments are available on Dryad (<https://datadryad.org/stash/landing/show?id=doi%3A10.5061%2Fdryad.t1g1jw2t>). The source code is available in GitHub (<https://github.com/luyinghms/Proteasome-FEL-model.git>)"

Figure 1. The architectures of the proteasomal ATPase complex and its interaction with substrate. **A.** A schematic showing a half 26S proteasome engaged with an unfolded substrate through the PL1s (color loops) on the ATPase subunits with bound nucleotides (color blobs). The disengaged PL1 is marked in grey. A black arrow suggests the staircase architecture of the

ATPases. **B.** Upper panel: a linear view of the architecture of the ATPase complex in a translocating state E_{D1} . Each ATPase subunit (Rpt1~Rpt6) is shifted vertically according to the position of its PL1 relative to the core particle. An open interface is suggested by a large gap between subunits. Arrows indicate the displacements of the PL1s and the change of nucleotides in the E_{D1} -to- E_{D2} transition. See Fig. 1-fig. supp. 1 for a complete list of these identified states of the proteasome. The lower panel illustrates how the ATPase rearrangement in the E_{D1} -to- E_{D2} transition may drive substrate translocation: the PL1 on Rpt4 disengages from substrate and moves to the distal registry of the staircase. This change is accompanied by an axial movement of the PL1s on Rpt1/2/6/3 that still interact with the substrate towards the core particle to bring about axial stepping and translocation of approximate 2xAAs. **C.** The solvent-excluded surface area at the interfaces of the ATPase domains in the seven substrate-engaged proteasome structures, colored according to the nucleotide at each binding pocket. The dashed line separates the closed and open interfaces, as defined here.

The following figure supplements are available for figure 1:

Figure supplement 1: Schematics showing the key structural features and the nucleotide-binding status of the substrate-engaged proteasome identified in a cryo-EM study.

Figure supplement 2: Example structures of open and closed interfaces.

Figure 2. Parameterization of the nucleotide-dependent free-energy landscape. **A.** The interaction map of the residues on Rpt4 and Rpt3 with the bound ATP or ADP in the E_{D1} or E_{D2} states. Red: *cis*-interacting residues on Rpt3. Blue: *trans*-interacting residues on Rpt4. **B.** A schematic showing the parameterization strategy for the free energy (E_{total}) of the ATPase hexamer and valuation of the parameters. The molecular interactions underpinning the three energy terms are marked by different colors. “Open/Closed” refers to the status of an ATPase interface S^i . “Empty/Occupied/ADP/ATP/ATP-γS” refers to the status of the nucleotide-binding pocket in the *cis* ATPase C^i . See methods 9.3 for a detailed explanation. **C.** The FEL on the E_D-like and E_C-like conformations of the ATPase complex in three representative nucleotide-binding statuses. T/D/E: ATP/ADP/empty. A sketch for the ATPase architecture of each conformation is listed on the right. The ATPases Rpt6/3/4/5/1/2 are represented by squares from left to right;

1094 dashed squares = disengaged ATPases that are flanked by open interfaces (gap between squares);
1095 the axial position of a PL1 is indicated by the vertical shift of the corresponding ATPase square.
1096 The following figure supplements are available for figure 2:
1097 Figure supplement 1: Phosphate groups interact weakly with the proteasomal ATPases.
1098 Figure supplement 2: Root-mean-square deviation(RMSD) of the nucleotide-interacting residues
1099 in the cis pockets among different proteasomal states.

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Figure 3. Evaluation of the FEL model parameters. **A.** A schematic showing three categories of nucleotide pockets with their corresponding dissociation constants K_d . **B.** Single-molecule nucleotide-proteasome interaction assay. 200nM Alexa647-ATP (red pentagon) was mixed ATP, ADP or ATP- γS (white pentagons) as a competitor at different concentrations, incubated with surface-immobilized 26S proteasome which was labeled with a SNAP-tag dye. The degree of Alexa647-ATP-proteasome colocalization in a steady state was measured using a TIRF microscope. The colocalization ratio (CR) after normalization by the competitor-free condition was inversely regressed on the competitor concentration to obtain the relative inhibitor constant K_i (methods 8). ADP concentration is divided by 10 for presentation. The inset illustrates the experimental design. Error bars represent the standard deviation of three replicas. **C.** Single-molecule translocation assay. N-terminal cyclinB was conjugated with Dylight550-labeled

1114 ubiquitins (yellow disks) on lysines(18, 36, 64) and was incubated with surface-bound 26S
 1115 proteasome in a buffer containing 0.5mM ATP or with extra 0.8mM ADP or with extra 40μM
 1116 ATP-γS. About 100 single-molecule traces exhibiting processive deubiquitylation in each
 1117 condition were aligned by the time of substrate-proteasome encounter ($t=0$). The average
 1118 fluorescent-ubiquitin intensity on a substrate molecule at each time point was calculated. The
 1119 rate of translocation was calculated from the initial slope of the traces. The inset illustrates the
 1120 experimental design. **D.** Representative traces of the degradation of ubiquitylated cyclinB-iRFP
 1121 by 1.5nM purified 26S proteasome with ATP (rep1~3) or ADP in the buffer. **E.** Lineweaver-
 1122 Burk plot of the initial degradation rate (v_0) at varying concentrations of cyclinB-iRFP (Sub)
 1123 either with 0.5mM ATP (--) or with extra 0.8mM ADP (+ADP). **F.** The translocation rate of
 1124 cyclinB-iRFP was measured using the fluorescent degradation assay with 0.5mM ATP and
 1125 various concentrations of ADP-Mg²⁺. Error bars represent the standard deviation of 15
 1126 measurements. The red curve shows the prediction by the FEL model.

1127 The following figure supplements are available for figure 3:

1128 Figure supplement 1: A block diagram for simulating the dynamics of proteasome using the FEL
 1129 model.

1130 Figure supplement 2: Examples of single-molecule traces showing processive ubiquitin chain
 1131 removal.

1132 The following source data files are available for figure 3

1133 Source data 1: Degradation kinetics of ubiquitylated cyclinB-iRFP at various concentrations of
 1134 substrate and proteasome. Related to figure 3E

1135 Source data 2: Degradation kinetics of ubiquitylated cyclinB-iRFP in the presence of 500μM
1136 ATP and various concentrations of ADP. Related to figure 3F
1137

1139

Figure 4. Evaluating the FEL-predicted degradation kinetics. **A.** Examples of simulated kinetics of translocation on individual proteasome particles under indicated nucleotide conditions. **B.** The translocation rate of cyclinB-iRFP measured at various concentrations of ATP-Mg²⁺, in comparison with the FEL-model prediction (red curve) based on the determined parameters. **C.** Same as in **B**, but measured with 0.5mM ATP and various concentrations of ATP-γS. Inset: translocation rate vs. ATP-hydrolysis activity as predicted by the FEL model at 0.5mM ATP and different concentrations of ATP-γS. **D.** Same as in **B**, but measured with 15mM ATP and various concentrations of ATP-γS. X-axis is the ratio between ATP-γS and ATP concentrations; Y-axis

1148 is the normalized translocation rate. The result in **C** is overlaid as a comparison. Inset: the
1149 absolute translocation rate vs. ATP- γ S concentrations.

1150 The following figure supplements are available for figure 4:

1151 Figure supplement 1: The workflow of this study, including the observations used for model
1152 construction, experimental validations of the simulated ATPase dynamics, and the major insights
1153 into the ATPase mechanism

1154 Figure supplement 2: High ATP concentration does not cause proteasome disassembly.

1155 Figure supplement 3: High concentration of Mg^{2+} alone does not affect the degradation kinetics.

1156 Figure supplement 4: High ATP concentration does not lead to partial protein degradation or
1157 uncoupling between ubiquitylation and degradation.

1158 Figure supplement 5: High ATP concentration does not inhibit the ATPase activity of
1159 proteasome.

1160 The following source data files are available for figure 4

1161 Source data 1 and 2: Degradation kinetics of ubiquitylated cyclinB-iRFP in the presence of
1162 various concentrations of ATP. Related to figure 4B

1163 Source data 3: Degradation kinetics of ubiquitylated cyclinB-iRFP in the presence of 500 μ M
1164 ATP and various concentrations of ATP- γ S. Related to figure 4C,D

1165 Source data 4: Degradation kinetics of ubiquitylated cyclinB-iRFP in the presence of 15mM
1166 ATP and various concentrations of ATP- γ S. Related to figure 4D

1167

Figure 5. Evaluating the FEL predictions for structurally-stable substrates. **A.** The initial degradation rate of ubiquitylated cyclinB-DHFR-iRFP by purified 26S proteasome with or without 0.8mM folic acid (FA), overlaid with the FEL model prediction. The inset shows the

1173 effective length of substrate peptide degraded by consuming one ATP molecule, as predicted by
1174 the FEL model. The normalized degradation rate is shown in **B**. Error bars represent the
1175 standard deviation of 15 measurements. **C**. Examples of simulated translocation kinetics when
1176 the ATPases encounter an unfolding-resistant domain at $t=0$, assuming substrate escape occurs at
1177 $Y=0$. **D**. Schematic showing the reactions in the competition assay for determining the residence
1178 time of an unfolding-resistant substrate on proteasome. R: cyclinB-iRFP reporter; C: cyclinB-
1179 DHFR-iRFP^{dark} competitor. **E**. Representative traces of the competition assay in D. **F**.
1180 Experimental values of the residence time of cyclinB-DHFR(MTX)-iRFP under indicated
1181 nucleotide conditions, in comparison with FEL model predictions with the peptide track length
1182 L_0 from 20 to 30AAs. Error bars represent the standard deviation of 5 measurements.

1183 The following figure supplements are available for figure 5:

1184 Figure supplement 1: Folic acid slows down the degradation of cyclinB-DHFR-iRFP, but not
1185 cyclinB-iRFP.

1186 Figure supplement 2: CyclinB-DHFR-iRFP was completely degraded by the proteasome in the
1187 presence of folic acid.

1188 Figure supplement 3: The degradation rate of cyclinB-DHFR-iRFP is inversely proportional to
1189 the folic acid concentrations.

1190 Figure supplement 4: The rate of ATP hydrolysis vs. ATP concentration as predicted by the FEL
1191 model.

1192 Figure supplement 5: Sensitivity of the simulated translocation rates to parameter variations.

1193

1194 The following source data files are available for figure 5:

1195 Source data 1: Degradation kinetics of ubiquitylated cyclinB-DHFR-iRFP in the presence of
1196 various concentrations of ATP without folic acid. Related to figure 5A,B

1197 Source data 2: Degradation kinetics of ubiquitylated cyclinB-DHFR-iRFP in the presence of
1198 various concentrations of ATP with folic acid. Related to figure 5A,B

1199 Source data 3: Degradation kinetics of ubiquitylated cyclinB-iRFP in the presence of cyclinB-
1200 DHFR-iRFP^{dark} as competitor, with either ADP or ATP- γ S in the buffer. Related to figure 5F

1201

Figure 6. Organization of proteasomal conformations in dynamical space. **A.** A diagram showing the steady-state transitions between the ATPase complex conformations in the FEL model. Each node represents a unique hexamer conformation, whose size is in proportion to its probability of occupancy in a steady state. Yellow and purple nodes are the E_D-like and E_C-like conformations respectively, with the corresponding ATPase architecture as presented in Fig. 2C. The labels of the conformations that have not yet been experimentally identified are bracketed. The width of a grey line is proportional to the total rate of forward and backward transitions

1211 between two conformations; and a dashed arrow represents the net transition rate and is colored
1212 according to the direction of substrate translocation associated with this conformational change.
1213 Minor conformations are randomly distributed in the graph. Supp. file 1 contains a full list of the
1214 conformations and their transition rates. The transition diagram without the hydrolysis activity of
1215 Rpt3 is shown in **B. C.** The mechanism of the cooperative movements of proteasomal ATPases
1216 during substrate translocation. The FELs on three conformations (the E_{D1} - and E_{D2} -equivalent
1217 and an E_C) are illustrated for four nucleotide statuses in a typical E_{D1} - to- E_{D2} transition (Fig. 6-fig.
1218 supp. 2). The ATPase architecture of each conformation is presented as in Fig. 2C. Arrows
1219 indicate the major conformation transitions upon the change of the nucleotide status and the
1220 associated FEL.

1221 The following figure supplements are available for figure 6:

1222 Figure supplement 1: The rates of substrate translocation and ATP hydrolysis for a Walker-B
1223 mutant proteasome.

1224 Figure supplement 2: The typical sequence of elementary steps underlying the E_{D1} -to- E_{D2} state
1225 transition.

1226 Figure supplement 3: The typical sequence of elementary steps underlying the E_{D1} -to- E_C state
1227 transitions.

1228 Figure supplement 4: The Lid-ATPase interaction may account for the observed dissymmetry in
1229 conformational occupancies in previous cryo-EM studies.

1230 Figure supplement 5: The Lid-ATPase interaction may account for the different growth
1231 phenotypes of yeast Walker-B mutants.

1232 Figure supplement 6: Global dynamical space with heterogeneous ATPase parameters.

1233

1234 Figure legends for figure supplements:

1235 **Figure 1-figure supplement 1.** Schematics showing the key structural features and the
1236 nucleotide-binding status of the substrate-engaged proteasome identified in a cryo-EM study.
1237 Each ATPase subunit is arranged vertically according to the order, or the approximate distance,
1238 of its pore-1 loop to the 20S core particle. An open interface is represented by a large gap. The
1239 Rpt2-Rpt6 interface is marked separately. Subunits that are disengaged from substrate are blank-
1240 and-white. The engage/disengage status is predicted according to the proximity of pore-1 loop to
1241 the central axis in the $E_{A1/2}$ and E_B states which do not exhibit substrate density in the
1242 translocation channel.

1243 **Figure 1-figure supplement 2. A.** The closed interface of the ATPase domains of Rpt3-Rpt4 in
1244 the E_{D1} cryo-EM state. Green: Rpt3; Cyan: Rpt4; Red: ATP; Arginine fingers on Rpt4 were
1245 shown with sticks. **B.** The open interface of Rpt3-Rpt4 in the E_{D2} cryo-EM state. Red: ADP. **C.**
1246 Open and closed structures were aligned by Rpt3(Green). Cyan: Rpt4 in E_{D1} ; light cyan: Rpt4 in
1247 E_{D2} .

1248 **Figure 2-figure supplement 1. Phosphate groups interact weakly with the proteasomal**
1249 **ATPases.** The degradation rate of ubiquitylated cyclinB-iRFP was measured at different
1250 phosphate concentrations, normalized by the rate at zero phosphate. The red curve is a fitting
1251 using the inhibitor equation to obtain the K_i value of 70mM for phosphate. See methods 6 and
1252 Fig. 3 for details on the activity assay.

1253 **Figure 2-figure supplement 2. Root-mean-square deviation(RMSD) of the nucleotide-**
1254 **interacting residues in the *cis* pockets among different proteasomal states.** The RMSD of the
1255 nucleotide-interacting residues in the *cis* binding pockets among different proteasomal states was
1256 analyzed as described in methods 1. The first three comparisons were gated according to whether
1257 a pocket changed from ‘occupied’ to ‘empty’ or *vice versa*. The last two compare the pocket
1258 geometry in the E_B , $E_{C1,2}$ and $E_{D1,2}$ states which have substrate peptide in the ATPase channel

with the pocket geometry in the EA1 or ED1 state. In the last analysis, only pockets with identical nucleotide status (“occupied” or “empty”) are compared. The figure shows the box plot for the RMSD distributions.

Figure 3-figure supplement 1. A block diagram for simulating the dynamics of proteasome using the FEL model. See methods §9 for a detailed description of the process.

Figure 3-figure supplement 2. Examples of single-molecule traces showing processive ubiquitin chain removal. Purified cycB_NT(K18,36,64) was ubiquitylated by APC-Cdh1 and E2 UbcH10 with Dylight550-Ub and was subjected to single-molecule proteasome assay as described in methods §7. Four traces with the signature of processive ubiquitin chain removal are presented as examples.

Figure 4-figure supplement 1. The workflow of this study, including the observations used for model construction, experimental validations of the simulated ATPase dynamics, and the major insights into the ATPase mechanism.

Figure 4-figure supplement 2. High ATP concentration does not cause proteasome disassembly. Purified human 26S proteasome was incubated with ATP-Mg²⁺ at indicated concentrations. After incubation, the sample was fixed with 1mM BS3 (bis(sulfosuccinimidyl)suberate) for 30 minutes and was analyzed on a native gel in the absence of nucleotide.

Figure 4-figure supplement 3. High concentration of Mg²⁺ alone does not affect the degradation kinetics. The quantitative degradation assay of ubiquitylated cyclinB-iRFP was performed as described in methods with 0.5mM ATP, with or without an extra 15mM MgCl₂. Two substrate concentrations were tested.

Figure 4-figure supplement 4. High ATP concentration does not lead to partial protein degradation or uncoupling between ubiquitylation and degradation. Ubiquitylated cyclinB-iRFP was radiolabeled with ³³p at both the N- and C-terminus using protein kinase A. The degradation assay was performed as described in methods and was sampled at indicated time

points and analyzed by autoradiography. U: unubiquitylated cyclinB-iRFP. “*”: radiolabeled securin was added as a control for loading and nonspecific dephosphorylation.

Figure 4-figure supplement 5. High ATP concentration does not inhibit the ATPase

activity of proteasome. The rate of ATP hydrolysis of purified 26S proteasome was measured in the presence of varying concentrations of ATP-Mg²⁺ using a Malachite green assay²⁷.

Denatured ovalbumin was used as a generic substrate for proteasome as described previously⁶⁴.

Error bars represent three experimental replicas.

Figure 5-figure supplement 1. Folic acid slows down the degradation of cyclinB-DHFR-

iRFP, but not cyclinB-iRFP. 100nM ubiquitylated cyclinB-DHFR-iRFP was tested in the quantitative degradation assay with 2nM proteasome at various concentrations of folic acid(A) or methotrexate(C) as indicated in the legend. Results for cyclinB-iRFP under identical conditions were shown in B and D.

Figure 5-figure supplement 2. CyclinB-DHFR-iRFP was completely degraded by the

proteasome in the presence of folic acid. Ubiquitylated cyclinB-DHFR-iRFP was radiolabeled with ³³P at both the N- and C-terminus using protein kinase A and was subject to degradation by 2nM purified human 26S proteasome as described in fig. 5-fig. supp. 1. +FA: in the presence of 800μM folic acid (+FA). Samples were analyzed by autoradiography which was intentionally overexposed to reveal any partially-degraded products.

Figure 5-figure supplement 3. The degradation rate of cyclinB-DHFR-iRFP is inversely

proportional to the folic acid concentrations. The degradation rate of cyclinB-DHFR-iRFP was measured at various folic acid concentrations as described in methods. Its inverse was plotted against the folic acid concentration.

Figure 5-figure supplement 4. The rate of ATP hydrolysis vs. ATP concentration as predicted by the FEL model.

The predicted rate of ATP hydrolysis per ATPase hexamer was plotted against the concentration of ATP in the FEL simulation as described in methods §9.5.

Figure 5-figure supplement 5. Sensitivity of the simulated translocation rates to parameter variations. A parameter is either increased or decreased by 30%, except for k_{on} which is varied by 3 fold due to the large uncertainty in its estimation. The simulated translocation rates under various nucleotide conditions in this study were compared with the original, and the mean deviation over the original translocation rate is presented. See methods 9.6.

Figure 6-figure supplement 1. The rates of substrate translocation and ATP hydrolysis for a Walker-B mutant proteasome. The translocation rate and ATPase activity were calculated using the FEL model in which Rpt3's ATP hydrolysis activity was abolished (WBM). The results are compared with those of the wild-type (WT) proteasome.

Figure 6-figure supplement 2. The typical sequence of elementary steps underlying the E_{D1} -to- E_{D2} state transition. In a simulation of the FEL model, the probabilities of all the pathways that lead to the E_{D1} to E_{D2} transition were calculated. The pathway with the highest probability is presented. This pathway is also embedded in most other E_{D1} - E_{D2} pathways. Irreversible steps are indicated by a single arrow and reversible steps are indicated by double arrows.

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Figure 6-figure supplement 5. The Lid-ATPase interaction may account for the different growth phenotypes of yeast Walker-B mutants. The translocation rate was calculated for each

Walker-B mutant proteasome (ATP hydrolysis set to zero) using the FEL model involving the ATPase-Lid interaction as described in methods §9.5. The growth phenotype of each Walker-B mutant is adapted from a previous study; “++++”: healthy; “+++”: slightly sick; “++”: sick; “+”: near-lethal; “-”: lethal¹¹. Varying the ATPase-Lid interaction from 1.6 to 4.0 kcal/mol gave qualitatively-similar results.

Figure 6-figure supplement 6. Global dynamical space with heterogeneous ATPase parameters. In the simulation, we let the basal energy E_b^i of the i^{th} ATPase = $\alpha \times \delta^i$. δ^i is a random number from 0 to 1 and α is a scaling constant. In **A**, we varied the value of α while fixing the ratios of E_b^i . In this example, $\delta^i = (0.45, 0.15, 0.83, 1.0, 0.68, 0.88)$. **B**. Two cases with alternative δ^i and the same α . **C**. The same E_b setting as in A(middle), but including the Lid-ATPase interaction as in fig.6-fig. supp. 4. The results are presented as in Fig. 6.

Legend for supplementary files 1:

1. ATPase conformations and steady-state occupancies

“Close interface”: the 6 digits indicate the Rpt6-Rpt3, Rpt3-Rpt4, Rpt4-Rpt5, Rpt5-Rpt1, Rpt1-Rpt2, Rpt2-Rpt6 interfaces. “0”: open; “1”: closed

“Engaged ATPases”: the 6 digits indicate Rpt6, Rpt3, Rpt4, Rpt5, Rpt1, Rpt2. “0”: disengaged subunit; “1”: engaged subunit.

“PL1 registry”: the 6 digits indicate the PL1s on Rpt6, Rpt3, Rpt4, Rpt5, Rpt1, Rpt2. “1~5”: part of the staircase architecture. “1” is closest to the CP; and “5” is farthest from the CP. “7”: disengaged PL1 at the top registry.

“Steady-state occupancy (%)”: the steady-state occupancy of each conformation in a FEL simulation.

2. Steady-state transition rates among ATPase conformations in the FEL model.

1361 “Total transition”: the total rate of transitions from conformation 1 to conformation 2 and reverse.
1362 Numbers are normalized by the highest value set to 100.

1363 “Net transition”: the absolute value of the rate difference between $\text{conf1} \rightarrow \text{conf2}$ and
1364 $\text{conf2} \rightarrow \text{conf1}$ transitions. Numbers are normalized by the same factor as above.

1365 “Translocation”: F: the net effect of conf1 - conf2 transitions is a forward translocation of
1366 substrate; “B”: the net effect is a backward translocation; “N”: this conformational transition
1367 does not lead to translocation.

1368

1369

Figure 1-figure supplement 1. Schematics showing the key structural features and the nucleotide-binding status of the substrate-engaged proteasome identified in a cryo-EM study. Each ATPase subunit is arranged vertically according to the order, or the approximate distance, of its pore-1 loop to the 20S core particle. An open interface is represented by a large gap. The Rpt2-Rpt6 interface is marked separately. Subunits that are disengaged from substrate are blank-and-white. The engage/disengage status is predicted according to the proximity of pore-1 loop to the central axis in the $E_{A1/2}$ and E_B states which do not exhibit substrate density in the translocation channel.

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Figure 2-figure supplement 2. Root-mean-square deviation(RMSD) of the nucleotide-interacting residues in the *cis* pockets among different proteasomal states. The RMSD of the nucleotide-interacting residues in the *cis* binding pockets among different proteasomal states was analyzed as described in methods 1. The first three comparisons were gated according to whether a pocket changed from ‘occupied’ to ‘empty’ or *vice versa*. The last two compare the pocket geometry in the EB, EC1,2 and ED1,2 states which have substrate peptide in the ATPase channel with the pocket geometry in the EA1 or ED1 state. In the last analysis, only pockets with identical nucleotide status (“occupied” or “empty”) are compared. The figure shows the box plot for the RMSD distributions.

Figure 3-figure supplement 1. A block diagram for simulating the dynamics of proteasome using the FEL model. See methods §9 for a detailed description of the process.

Structural rules

- The staircase architecture of PL1s
- 2~3 open interfaces in each ATPase complex
- At least 1 disengaged ATPase per complex; disengaged ATPases move to the top of the staircase
- ATPase interfaces adopt approximate binary states
- Translocation by ATPase power stroke

Kinetic measurements

- Nucleotide-proteasome interaction
- Degradation kinetics at different ADP concentrations
- ATP hydrolysis rate (published) and etc.

The FEL Model

Conformational coordinates
The FEL model parameters

ATPase mechanisms

- The cooperativity mechanism of the ATPases (section III)
- ATP-hydrolysis model for the ATPase complex (section IV)
- How are conformational changes coupled to the ATP cycle? (section IV)
- The mechanism of translocation directionality (section IV)
- And etc...

Global ATPase dynamics

Predictions

Kinetics-related validations

- Degradation kinetics at different ATP and ATP-γS concentrations: Quantitatively consistent
- Degradation kinetics of difficult-to-unfold (DHFR) substrate: Quantitatively consistent
- Kinetics of backward translocation: Quantitatively consistent
- Activities of Walker-B mutant proteasomes: Qualitatively consistent

Structure-related validations

- Steady-state conformational occupancies of wild-type and Walker-B mutant proteasomes: Qualitatively consistent
- Correlation between ATPase conformation and nucleotide status: Consistent

Approximations & assumptions

- The six ATPases share identical parameters
- The detailed process of conformational change is treated “adiabatic”; the rate is described by the Arrhenius equation
- The resistive force from substrate during translocation is approximated by two constants
- The effect of Lid-ATPase interaction is simplified as stabilizing S_A -like conformations.
- Phosphate release is assumed fast enough so that ADP-Pi state can be ignored.

Figure 4-figure supplement 1. The workflow of this study, including the observations used for model construction, experimental validations of the simulated ATPase dynamics, and the major insights into the ATPase mechanism.

Figure 4-figure supplement 2. High ATP concentration does not cause proteasome disassembly. Purified human 26S proteasome was incubated with ATP-Mg²⁺ at indicated concentrations. After incubation, the sample was fixed with 1mM BS3 (bis(sulfosuccinimidyl)suberate) for 30 minutes and was analyzed on a native gel in the absence of nucleotide.

Figure 4-figure supplement 4. High ATP concentration does not lead to partial protein degradation or uncoupling between ubiquitylation and degradation. Ubiquitylated cyclinB-iRFP was radiolabeled with ^{33}p at both the N- and C-terminus using protein kinase A. The degradation assay was performed as described in methods and was sampled at indicated time points and analyzed by autoradiography. U: unubiquitylated cyclinB-iRFP. “*”: radiolabeled securin was added as a control for loading and nonspecific dephosphorylation.

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