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Towards the understanding of molecular motors and its relationship with local unfolding

Zahra Alavi[1], Nathalie Casanova-Morales[2], Diego Quiroga-Roger[3] and Christian A.M. Wilson[3]

[1]Department of Physics, Loyola Marymount University, Los Angeles, CA, USA; [2]Facultad de Artes Liberales, Universidad Adolfo Ibáñez, Santiago, Chile and [3]Biochemistry and Molecular Biology Department, Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, Santiago, Chile

Review

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Corresponding author:
Christian A.M. Wilson;
Email: yitowilson@gmail.com

Z.A. and N.C.-M. contributed equally to this paper.

Abstract

Molecular motors are machines essential for life since they convert chemical energy into mechanical work. However, the precise mechanism by which nucleotide binding, catalysis, or release of products is coupled to the work performed by the molecular motor is still not entirely clear. This is due, in part, to a lack of understanding of the role of force in the mechanical–structural processes involved in enzyme catalysis. From a mechanical perspective, one promising hypothesis is the Haldane–Pauling hypothesis which considers the idea that part of the enzymatic catalysis is strain-induced. It suggests that enzymes cannot be efficient catalysts if they are fully complementary to the substrates. Instead, they must exert strain on the substrate upon binding, using enzyme-substrate energy interaction (binding energy) to accelerate the reaction rate. A novel idea suggests that during catalysis, significant strain energy is built up, which is then released by a local unfolding/refolding event known as 'cracking'. Recent evidence has also shown that in catalytic reactions involving conformational changes, part of the heat released results in a center-of-mass acceleration of the enzyme, raising the possibility that the heat released by the reaction itself could affect the enzyme's integrity. Thus, it has been suggested that this released heat could promote or be linked to the cracking seen in proteins such as adenylate kinase (AK). We propose that the energy released as a consequence of ligand binding/catalysis is associated with the local unfolding/refolding events (cracking), and that this energy is capable of driving the mechanical work.

General overview of protein structure–function relationships

In the last 70 years, many studies focused on the protein structure–function relationships have been achieved to a detailed level by high-resolution structures determined by X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, Cryo-electron microscopy and Alpha fold (Henzler-Wildman *et al.,* 2007; Klinman and Hammes-Schiffer, 2013; Nogales, 2016; Jumper *et al.,* 2021). This is reflected in the large number of protein structures (200,069 until January 10, 2023, associated with functional studies that are now included in the protein data bank (PDB), demonstrating the importance and validity of the relationship between the protein structure and functional researches.

Folding and conformational changes in proteins

The global spatial arrangement of atoms in a protein is called protein conformation. Catalysis is not only determined by chemicals steps, but also by conformational changes in protein structure. Proteins must adopt a specific conformation, or in other words, a specific three-dimensional structure (native protein) to be catalytically active (Anfinsen *et al.,* 1954; Carrion-Vazquez *et al.,* 1999; Klinman and Hammes-Schiffer, 2013). In fact, proteins can be found mostly folded in physiological conditions. The stability of native proteins in folded state is only marginally higher compared to the unfolded state (Taverna and Goldstein, 2002; Magliery, 2015), as the difference in Gibbs free energy (ΔG) separating folded and unfolded states is only about 5 to 15 kcal/mol (Creighton, 1990; Voet and Voet, 2010). Protein stability studies have usually been carried out by reversibly unfolding using denaturants (chaotropic agents such as guanidine chloride and urea), increasing or decreasing the temperature, varying the pH, applying high pressures, or cleaving disulfide bonds. These studies allow for the determination of the free energies necessary to maintain the folded state, the intermediate states if they exist, the folding energy landscape, and the population of the unfolded protein (Dill, 1985; Shea and Brooks, 2001).

Many biochemical reactions proceed via large conformational changes within or between interacting molecules. In the past, protein conformational changes were viewed as rigid body

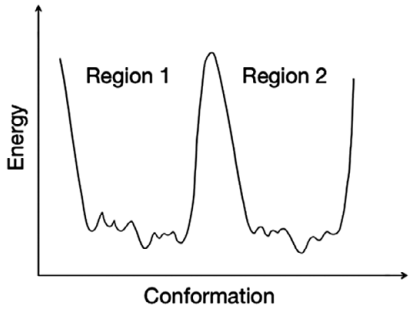

Figure 1. The many different conformational states produce a rocky landscape.

movements coupled to ligand binding with just one lowest free energy conformation in each condition (with and without ligand). One of the best examples is hemoglobin, where the conformational changes are coupled to the binding of an oxygen molecule in a remote site of the active site (allosterism) (Edelstein, 1975). However, the current concept of native proteins considers them as ensembles of related, interconverting, transient microstates that describe the canonical high-resolution structures observed by crystallography or NMR spectroscopy (Frauenfelder *et al.,* 1988; Hilser *et al.,* 2006; Fenimore *et al.,* 2013). Thus, protein movements are consequence of a collection of microstates that occur at different time scales, as seen in Figure 1.

The conformations existing under a given set of conditions are usually the ones that are thermodynamically the most stable, having similar ΔG (Creighton, 1990; Voet and Voet, 2010). The existence of multiple stable conformations reflects the changes that occur in proteins as they bind to other molecules or when they catalyze reactions, validating the idea that many of the physical and functional properties of proteins (stability, solubility, binding of ligands) are influenced by the same structural fluctuations that give rise to the ensemble without a ligand (Hilser *et al.,* 2006; Klinman and Hammes-Schiffer, 2013).

Ligand-binding and conformational changes in catalysis

Protein conformational changes play a crucial role in catalysis, enabling proteins to bind to ligands, form oligomers, and perform mechanical work (Koshland, 1958; Whitford *et al.,* 2007; Olsson and Wolf-Watz, 2010). These conformational changes can occur in time scales from milliseconds to nanoseconds (Henzler-Wildman *et al.,* 2007) and involve domain movements. According to the 'induced fit' theory (Koshland, 1958), substrate binding triggers a large conformational change in the enzyme, leading to the correct positioning of the residues involved in catalysis and the release of the product. In this view of enzyme catalysis, binding interactions between the substrate and the enzyme provoke motions in enzyme's structure that bring the substrate into the transition state, facilitating and pushing forward the catalytic process along its reaction coordinate (Bustamante *et al.,* 2004; Voet and Voet, 2010). Kinases are generally viewed as good examples of the induced-fit mechanism (Koshland, 1958; Schulz *et al.,* 1990; Choi and Zocchi, 2007).

Another view of enzyme catalysis, according to the Haldane–Pauling hypothesis (Pauling, 1946; Haldane, 1965), considers the idea that part of the enzymatic catalysis is 'strain-induced' (also called geometric destabilization theory (Jencks, 1975). It suggests that enzymes cannot be efficient catalysts if they are fully complementary to the substrates and they must exert strain on the substrate upon binding. Furthermore, it suggests that in order to catalyze reactions, an enzyme must be complementary to the transition state species, and not to the substrate molecule itself in its normal configuration. This hypothesis considers that the energy derived from the enzyme-substrate interaction (the binding energy) is used to accelerate the rate of the reaction. Experimental evidence of strain-induced catalysis has been indirect (Amyes and Richard, 2013). One example is using catalytic antibodies. They recognize and bind non-covalently to their complementary antigen, then catalyze bond-breaking or other chemical reactions involving the antigen (Hanson *et al.,* 2005). In those works, they used molecules that were analogs to the transition state which bind more tightly to the antibody than its actual antigen or substrate (Pollack *et al.,* 1986; Tramontano *et al.,* 1986; Savinov *et al.,* 2003). Another example is the study of the strain in the carbonyl bond of the substrate of beta-lactamase (Hokenson *et al.,* 2000). Class A beta-lactamases hydrolyze penicillins and other beta-lactams via an acyl-enzyme catalytic mechanism. Comparison of the beta-lactam carbonyl stretch frequency in the free and enzyme-bound substrate revealed an average decrease of 13 cm^{-1} in its frequency, indicating substantial strain/distortion of the lactam carbonyl when bound in the enzyme-substrate (ES) complex. The substrate binding to the active site induces substantial strain and distortion that contributes significantly to the overall rate enhancement in beta-lactamase catalysis (Hokenson *et al.,* 2000). Although these data help us to understand strain-induced catalysis, they are not enough to quantify the contribution of strain in catalysis. It is also not clear how this event is linked with the conformational changes coupled to ligand binding.

Molecular motors

Enzymes catalyze chemical reactions that occur in life, playing a key role in almost all biological events as they accelerate the rate of chemical reactions by lowering the energy barrier of the conversion of the reactants into products (Voet and Voet, 2010; Cornish-Bowden, 2012).

Motor enzymes use the energy of nucleotide binding, hydrolysis, or product release to generate mechanical work. Thus, these motor enzymes must couple one or more of those chemical steps to mechanical transitions. There are many proteins that behave as molecular machines. For example, myosin, kinesin, and dynein families use ATP hydrolysis as a source of energy to move along a track. Also, polymerases must utilize part of the chemical energy derived from the polymerization reaction to move along the DNA or RNA in a unidirectional manner (Bustamante *et al.,* 2004). Helicases hydrolyze ATP to translocate along DNA, unwinding it into its complementary strands (Cheng *et al.,* 2007). Some viruses use ATP hydrolysis to pack the DNA inside their capsid against considerable entropic, electrostatic, and elastic forces (Smith *et al.,* 2001). In the case of translocation, a molecular motor uses ATP binding/hydrolysis to mechanically pull polypeptide chains (a flexible, irregular polymer) across membranes.

All these motors move along stiff tracks such as double-stranded (ds) DNA or microtubules, where the track persistence length (P; or bending stiffness of a polymer) is an order of magnitude larger than the step size of the motor (Maillard *et al.,* 2011).

Brownian ratchet versus power stroke model

There are two general models to explain how molecular motors operate: the Brownian ratchet and the Power stroke model. In the

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Brownian ratchet model, the motor uses nucleotide binding and/or hydrolysis to direct and rectify its Brownian motion, with a net movement in one direction (Astumian, 1997). In the power stroke model, the motor utilizes the energy of ATP binding and/or hydrolysis or product release to drive the motion (Smith *et al.,* 2001). A typical Brownian motion ratchet mechanism molecular motor is the *E. coli* RNA polymerase with a work (stall force times step size) value close to 2 k_BT (k_B is the Boltzmann constant and T is the temperature) at stall force (force at which the velocity of the motor drops to zero); (Neuman *et al.,* 2003). In the case of the bacteriophage Phi29 (a power stroke motor), the work value is about 10 k_BT (Moffitt *et al.,* 2009). It is considered a power stroke model when the work is higher than 5 k_BT at the stall force (Bustamante *et al.,* 2004). How do motors convert the chemical energy into mechanical work or movement? What is the mechano-chemistry of the motor? The force can be considered as a product of the nucleotide binding/hydrolysis/product release cycle, and the velocity of a motor often depends on the external force and is related to the mechanism of the motor (Bustamante *et al.,* 2004). The rate of the motor will be force-dependent if, in the conditions of the experiments, the movement is the rate-limiting step. By systematically varying the concentration of ATP, or its hydrolysis products (ADP, Pi) at different forces, it is possible to determine the force-generating step during the chemical cycle of ATP hydrolysis and to study the mechano-chemistry of molecular motors (Wang *et al.,* 1998; Bustamante *et al.,* 2004). These studies have been done in many systems. For example, it has been shown that the rate constants of certain sub-steps in the mechanochemical cycle of molecular motors are force-dependent (or torque-dependent in rotary motors) as seen for Myosin and F1-ATPase (Oguchi *et al.,* 2008; Sellers and Veigel, 2010; Adachi *et al.,* 2012; Watanabe *et al.,* 2012; Cossio *et al.,* 2015; Volkán-Kacsó and Marcus, 2015; Houdusse and Sweeney, 2016). Molecular motors are enzymes. Thus to understand the mechanisms that govern their function we should focus on how enzymes work.

Importance of forces in mechanochemical processes

Different processes inside the cell, such as chromosomal segregation, DNA transcription, DNA replication, the formation and liberation of vesicles, the packing of DNA during viral replication, and the translocation of protein through channels, are mechanical processes (Schekman, 1994; Bustamante *et al.,* 2004; Cecconi *et al.,* 2005; Zhang *et al.,* 2009). As mentioned before, protein folding and unfolding, conformational changes, and catalysis also represent good examples of mechanical processes studied by the application of a mechanical force. One of the advantages of studying the reactions with a focus on force is that the reaction coordinate is an easily quantifiable physical parameter. The kinetics of a reaction can be studied by the application of a mechanical force (Bell, 1978). An applied mechanical force affects the free energy, equilibrium, and rate of a reaction occurring along a mechanical reaction coordinate, so thermodynamic and kinetic parameters could be determined at zero force extrapolating the information obtained at different forces. Recently, new transition state theories stemming from the application of Kramers's theory (Kramers, 1940) have emerged (Dudko *et al.,* 2008; Cossio *et al.,* 2016; Bullerjahn *et al.,* 2020). Another interesting and useful application of force in the study of conformational changes and catalysis, called 'the allosteric spring probe' (ASP), was developed by the group of G. Zocchi (Choi and Zocchi, 2007), using a guanylate kinase (GK) as a model. The novelty of this technique is in the attachment of a single-stranded DNA spring to a protein, followed by using complementary strands of different sizes to apply a mechanical force, generating useful information about the mechanism of the reaction and allowing to assess quantitatively the relevance of the induced-fit theory in this enzyme.

Force is also very important in many biological signaling processes. For example, in order to start the coagulation process the Von Willebrand factor unfolds via shear forces, and exposes the binding site to start the process (Zhang *et al.,* 2009). It has been shown that the folding force of SNARE proteins is enough to achieve membrane fusion in the vesicle release at the synapse (Gao *et al.,* 2012; Zhang *et al.,* 2016). Another study has shown that the refolding of the Top7 protein is enough to release a stall sequence from the ribosome (Goldman *et al.,* 2015). All these studies show that refolding force is crucial for biochemical processes in all cells.

Local unfolding/refolding (cracking)

In recent years, various studies have added a higher level of complexity to enzyme catalysis. These studies include computational molecular dynamics such as mixed Go model (Miyashita *et al.,* 2003; Whitford *et al.,* 2007), or laboratory bench experiments such as ITC (Isothermal Titration Calorimetry) (Schrank *et al.,* 2009). Entropy-promoting mutations (Olsson and Wolf-Watz, 2010) have also provided evidence of the existence of local unfolding/refolding events (also known as 'cracking') during catalysis (Klinman and Hammes-Schiffer, 2013; Schrank *et al.,* 2013). Adenylate kinase (AK) is an excellent example of a protein that undergoes cracking during catalysis, where significant strain energy (>20 kcal/mol) is built up during the reaction, and then released through a local unfolding event at the transition state. The protein then refolds at the downhill side of the activation energy barrier and the thermodynamic minimum is reached (Miyashita *et al.,* 2003; Whitford *et al.,* 2007; Olsson and Wolf-Watz, 2010). The novel Cracking idea points to make more complex the interconversion between the two simple rigid-body folded open and closed states. By means of connecting the initial and final states through a local unfolding and refolding event and adding another equilibrium step between the folded and unfolded states coupled to ligand binding or catalysis.

Another cracking event has been described for the SecA protein. SecA is a highly conserved and essential ATP-dependent motor protein of the bacterial Sec translocase machinery. This protein uses cracking to control its activation process in a precise manner and adopt alternate conformational states during the ATPase cycle (Keramisanou *et al.,* 2006). Moreover, evidence shows that in some catalytic reactions with conformational changes coupled to catalysis, part of the heat released results in a center-of-mass acceleration of the enzyme, raising the possibility that the heat released by the reaction itself could affect enzyme's integrity. Thus, it has been suggested that this released heat could be promoting or be linked with cracking seen in AK (Riedel *et al.,* 2015). This is because enough energy is released upon catalysis, and the enthalpy change (ΔH) of the reactions that proteins catalyze is high enough to unfold the protein (Riedel *et al.,* 2015).

Interestingly the theoretical ΔH in some reactions differs from the experimental one in the presence of enzymes (Table 1), with the experimental ΔH being lower. This difference may be due to some of the energy being used to perform work in the enzyme, such as moving the center of mass of the protein or locally unfolding some

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Table 1. Theoretical and experimental characterization of enthalpies

Enzyme	Reaction	$\Delta H_{theoretical}$ kcal/mol	$\Delta H_{experimental}$ kcal/mol	Conditions	References
Catalase	$H_2O_2(aq) \rightarrow H_2O + 1/2O_2$	−22.7	24 ±0.3	0.154 M NaCl+EDTA 0.001 M 25C 0.05% sodium pyrophosphate (pH 7.0)	(Sinha, 1972)
Hexokinase	Glucose + MgATP → Glucose6P + MgADP	−5.4	−10.76	0.05 M HEPES +0.05 M KCl + 0.02 M $MgCl_2$ + 0.009 M ATP (pH 7.0)	(Olsen, 2006)
Hexokinase1	Glucose + MgATP → Glucose6P + MgADP	−5.4	−5.1 ±0.2	0.05 M (buffer) + 0.200 M KCl + 0.005$MgCl_2$+ + 0.01 glucose +0.0001 ATP (pH 7.6)	(Bianconi, 2003)
Hexokinase2	Glucose + MgATP → Glucose6P + MgADP	−5.4	−3.3 ±0.3	0.05 M (buffer) + 0.200 M KCl + 0.005 M $MgCl^{2+}$ + 0.01 M glucose +0.0001 M ATP (pH 7.6)	(Bianconi, 2003)
Urease	$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$	−24.61	−14.9/−14.1	0.05 M HEPES +0.05 M Na_2SO_3 + 0.15 M NaCl (pH 7.0)	(Benini *et al.*, 2014)
Pyruvate carboxylase	piruvato + CO_2 + H_2O + ATP → oxalacetato + ADP + Pi + 2H +	42.5	21.4	0.02 M HEPES +0.004 M $NaHCO_3$ (pH 7.2)	(Kemp and Guan, 1999)

This table shows the enzyme and the reaction with which the theoretical ΔH enthalpy is calculated based on thermodynamical tables (Tinoco *et al.*, 2013). Experimental enthalpy $\Delta H_{experimental}$ is that which is measured in an experiment under the conditions specified by the studies referenced in the last column.

region of it (Riedel *et al.*, 2015). Overall, these findings highlight the complexity of enzyme catalysis and the role that cracking events may play in the process.

Dynamic of local unfolding and the generation of work

But how fast will the protein refold? For folding of a complete protein, the experimental and theoretical approaches predict a speed limit of approximately N/100 μs for a generic N-residue single-domain protein (Kubelka *et al.*, 2004; Dill *et al.*, 2008). Then, a small portion of the protein could fold very fast because of its size which is compatible with the catalysis timing. For example, each catalytic cycle in AK takes 25–40 ms and the size of the local unfolded region is around 20 amino acids, predicting a refolding time of 0.2 μs, one order of magnitude faster than the turnover number (Pelz *et al.*, 2016).

Despite the various hypotheses surrounding enzyme catalysis, there is still uncertainty about where the catalytic power of the enzyme resides. Most of these ideas have remained as hypotheses, partly due to the difficulty of designing experiments to demonstrate them. While the induced-fit mechanism is widely accepted (Klinman and Hammes-Schiffer, 2013), the 'strain-induced' idea remains largely hypothetical as bulk chemical experiments provide only indirect support for its validity (Bustamante *et al.*, 2004). Moreover, it is challenging to correlate conventional bulk experiments with *in silico* dynamic simulations to demonstrate the existence of cracking. Therefore, it is crucial to find a molecular description of the mechanisms by which enzymes achieve high catalytic efficiency coupled to conformational changes, demonstrating the importance of strain in these processes. A modern approach to address this question is to consider protein folding and unfolding, conformational changes, and catalysis as mechanical processes, where basic physical concepts such as force, torque, work, energy conversion efficiency, and mechanical advantage can be determined to describe them (Bustamante *et al.*, 2004).

Based on this knowledge, we could hypothesize that the energy released by binding/catalysis in an enzyme is coupled to local unfolding and the refolding is the process that allows the mechanical work.

Another indication of local unfolding is the existence of intermediates. Intermediates of the folding pathway are very common even for very small proteins (Rivera *et al.*, 2023b). Even for typical two-states proteins, if we change more parameters to unfold the protein we could see an intermediate (Rivera *et al.*, 2023b). These intermediates could be related to the local unfolding of proteins and may be a universal characteristic. In the past unfolding regions were seen as non-functional regions of the protein. Today the view is completely contrary. There are many proteins that have disordered regions that are essential for their functions. Such proteins are known as intrinsically disordered proteins (IDPs) (Oldfield and Dunker, 2014). It has been shown that these disordered regions can fold upon binding to another protein (Malagrinò *et al.*, 2020).

In our view of molecular motors, an unfolded region will be very important to perform work once it is re-folded. As previously mentioned, the refolding force is enough to perform work in some systems such as SNAREs, SecA, and IDPs which can fold under different conditions such as substrate binding, protein–protein interaction, and so on (Gao *et al.*, 2012; Goldman *et al.*, 2015).

Enzymes as minimal molecular motors and instruments to study them

Our approach is to consider enzymes as molecular motors converting chemical energy (either in the form of binding energy, chemical bond hydrolysis, or electrochemical gradients) into mechanical work through conformational changes and displacements (Bustamante *et al.*, 2004; Zocchi, 2018). The energy accumulated as a consequence of ligand binding or product release could be used to execute conformational changes during catalysis. We believe that some ATPases are minimum molecular motors, that the movement of domains coupled to catalysis can be considered as the simplest motor in nature and that with single molecule studies we can understand their behavior. By applying an external force over the moving domains, the ES complex formation, or the transition state attainment can be affected. So, if the conformational change is rate-limiting, the external force will significantly affect the rate of the

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reaction, providing direct evidence for the effect of strain in enzyme catalysis. Moreover, by varying the magnitude of the external force applied to the enzyme-substrate complex, it should be possible to estimate the maximum force generated within the complex. Therefore, by the optical tweezer single-molecule approach changes can be monitored as a function of velocity and force, allowing for the determination of the rate-limiting step of ligand binding and catalysis, and quantifying the force needed to stop catalysis ('stall force') (Bustamante *et al.,* 2004; Choi and Zocchi, 2007). Thus, this force can be correlated with the force that the enzyme exerts on the substrate, which in fact is the strain that the enzyme exerts on the substrate, according to the stress-induced catalysis hypothesis (Pauling, 1946; Haldane, 1965). Next, we will look at three proteins through the lense of cracking as models to study the force in unfolding events in molecular motors.

Aquifex aeolicus *adenylate kinase*

A suitable enzyme to study as a minimal molecular motor should involve a big conformational change upon substrate binding, it should display a correlation between turnover number and the fluctuations in the domain closure, the turnover number should not be too fast (slower than the detection system), and the molecule should be preferably a monomer (Figure 2*a*). In this context AK from the thermophilic organism *Aquifex aeolicus* and GK from *Mycobacterium tuberculosis* are good candidates. AK (EC 2.7.4.3) is a small (20–26 kDa) monomeric ubiquitous signal transducing enzyme that is responsible for maintaining cellular steady-state concentration of adenylate nucleotides in the cell (Schrank *et al.,* 2013). This enzyme has been studied in detail for decades and much is known about its structure, function, kinetics, and taxonomy. This enzyme catalyzes the reversible phosphoryl transfer reaction, using Mg^{2+} or Mn^{2+} as a cofactor, according to: $Mg^{2+} + ATP + AMP \leftrightarrow Mg^{2+} + 2ADP$ (Hamada *et al.,* 1985; Schulz *et al.,* 1990). From a structural point of view, this protein is modular and is formed by three subdomains: the core subdomain that is very important for the overall stability, the ATP and AMP binding subdomains that have the ATP and AMP binding sites, and the lid subdomain that increases catalytic efficiency by covering the binding sites. The X-ray crystal structures of AK have been solved in an open (substrate-free) and a closed (inhibitor-bound) conformation (Lienhard and Secemski, 1973; Müller and Schulz, 1992). Analysis of the three-dimensional structures of AK, in both the bound and unbound states, reveals that the lid subdomains and the ATP and AMP subdomains undergo a significant conformational change in response to substrate binding (Schulz *et al.,* 1990). This movement has been described as domain closure, which in the case of AK is 11 Å (Schulz *et al.,* 1990; Sinev *et al.,* 1996), being in the closed conformation where the phosphoryl transfer reaction occurs (Müller and Schulz, 1992; Ådén and Wolf-Watz, 2007) and as explained previously has been shown that it undergoes cracking. Since the resolution limit of the technique is at least 10 Å, AK is a good candidate for the study of its catalysis with the optical tweezers (Wilson *et al.,* 2013; Pelz *et al.,* 2016). AK has a catalytic constant (k_{cat}) of 40 sec^{-1} (40 catalysis events per second) which is well within the instrumental capabilities, since it is possible to determine cycles of catalysis of 40 events or less with our bandwidth of 1000 Hz. The k_{close} and k_{open} of this enzyme were determined to be 44 and 1600 Hz, respectively (Henzler-Wildman *et al.,* 2007). Some correlations between the enzyme flexibility and catalysis have already been described using bulk techniques (Antikainen and Martin, 2005). For instance, the work of Wolf-Watz *et al.* (2004) and Henzler-Wildman et al. (2007) found strong correlations between the k_{open} and the turnover number by comparing the mesophilic and the thermophilic AK. A work from (Ben Ishay *et al.,* 2012) using time-resolved FRET had been shown that AK can refold locally a domain of the protein within the dead time of the stopped-flow device, showing that this local refolding is below 5 ms. This shows that the protein could unfold and refold faster than the catalytic cycle. If this intermediate corresponds to the cracked region with faster techniques and/or transition paths analysis (Cossio *et al.,* 2018) we should be able to isolate this metastable state.

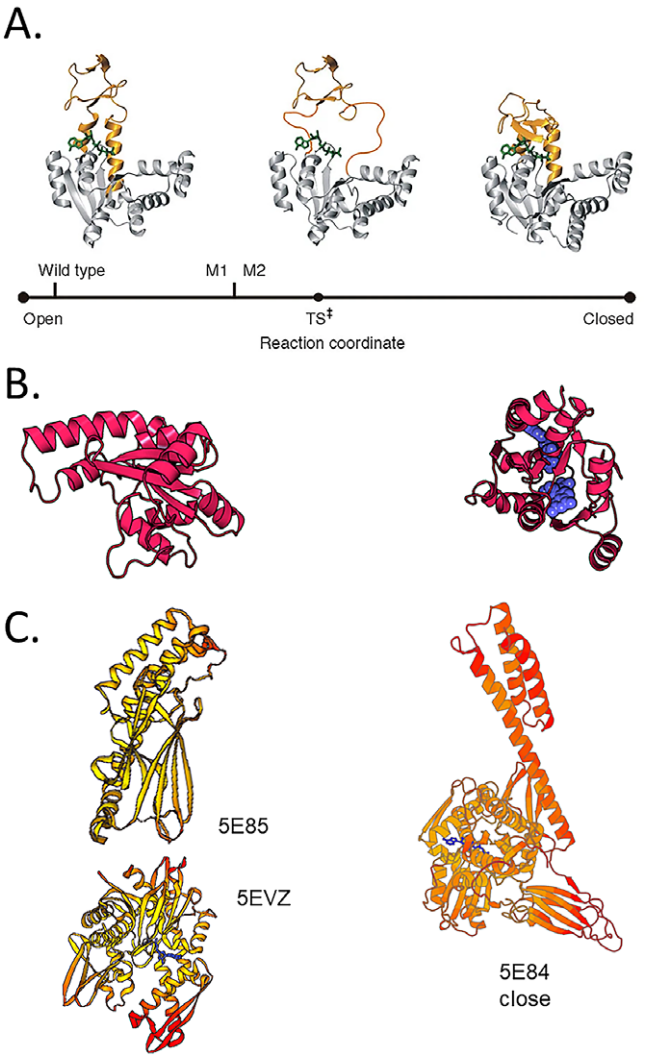

Figure 2. Single-molecule force spectroscopy techniques (open and closed conformations of the proteins to be studied). (*a*) Left: Open apo Aquifex AK (PDBid: 2RH5). Middle: Local unfolded or crack intermediate. Right: Close Aquifex AK in complex with the substrate analogue Zn^{2+}● Ap5A (light blue) (PDBid: 2RGX) (Olsson and Wolf-Watz, 2010). In yellow is the lid domain and in gray is the core of the protein. Reprinted by permission from Springer Nature. (*b*) Left: Open configuration GK (PDBid: 1ZNW). Right: Close configuration of GK (PDBid: 1LVG) upon substrate binding. Alpha helices are colored magenta and beta sheets are colored yellow. (Alavi, 2017). (*c*) Open configuration BiP in the presence of ADP (PDBids: 5EVZ and 5E85) and right. Close configuration of BiP in the presence of ATP (PDBid: 5E84). In blue is the NBD and orange is SBD (Pobre *et al.,* 2019). Images were made with 3D Protein imaging software (Tomasello *et al.,* 2020).

Another interesting feature of AK is that this enzyme has an important role in health. There exists a lot of experimental data

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validating the direct relationship between AK and human diseases. For example, knockout of the major human AK isoform (AK1) demonstrated a deficit in vascular and myocardial AK catalysis blunts energetic communication, AMP, and adenosine metabolic signal transduction. This compromises post-ischemic coronary reflow, providing evidence for the role of AK as a metabolic monitor supporting regulation of the reactive vascular response in the stressed heart (Dzeja *et al.,* 2007). AK1 deficiency has also been related to hemolytic anemia (Abrusci *et al.,* 2007). It has been observed that human AK isoform (AK4) is upregulated in lung adenocarcinoma compared with normal cells, suggesting that AK4 promotes malignant progression and recurrence by promoting metastasis in a transcription factor ATF3-dependent manner (Jan *et al.,* 2012). Therefore, understanding the mechanisms by which AK exerts its efficient catalysis is of great importance as it could help to unravel the causes and to develop therapies involving the AK function. In this respect, we have determined the sequence identity between *Aquifex aeolicus* adenylate kinase (Aquifex AK) and human AK, establishing that all of them have substantial identity (over 90% between Aquifex AK and Human AK1, AK2, and AK3 isoenzymes).

Mycobacterium tuberculosis *guanylate kinase*

The other protein of interest is GK which catalyzes the ATP-dependent phosphorylation of GMP into GDP (ATP + GMP ⇌ ADP + GDP) (Stehle and Schulz, 1992). In humans, GK is the only known enzyme responsible for GDP production and thus is essential for cellular viability and might be a potential target for cancer therapeutics (Khan *et al.,* 2019). This protein is around 24 kDa and 4 nm long, similar in size and conformation to AK. As seen in Figure 2*b*, GK's structure consists of a clamp-like cavity with three distinct regions: a central CORE domain, a LID which closes onto the CORE domain during catalysis and a GMP binding domain (GMP-BD). Additionally, a canonical P-loop motif is involved in ATP binding (Choi and Zocchi, 2007; Khan *et al.,* 2019).

Upon substrate binding the two lobes of the clamp close through an ∼1-nm conformational change, most of which is induced by GMP binding. This conformational change that is relatively large compared to the size of the enzyme involves several direct and indirect (via water molecules) interactions which bring the LID and the GMP-BD jointly toward the CORE region (Choi and Zocchi, 2007). This substrate-induced conformational change turns the enzyme from the open state to the close state, but it is not known if this conformational change takes place via cracking. In this view, the catalytic cycle of the enzyme can be described by the kinetic cycle of conformational changes, an idea that was proposed more than 50 years ago (Bliumenfel'd, 1971) and experimentally studied using NMR and Nanorheology (Eisenmesser *et al.,* 2002; Qu and Zocchi, 2013).

In 2007, Choi and Zocchi performed an elegant experiment on GK by inserting an externally controllable molecular spring on the protein (an allosteric spring probe, ASP). In this experiment, 60 mer DNA was used as the probe which in ss form exerts essentially no tension but significantly rigidifies upon hybridization with a complementary strand and therefore exerts a mechanical stress on the protein (Choi and Zocchi, 2007).

They found that when the open structure is favored through mechanical stress the binding affinity for GMP is drastically reduced, whereas the binding affinity for ATP and the catalytic rate were unaffected. This result shows that GMP binding does not allosterically control ATP binding but must allosterically control catalysis or at least the ATP hydrolysis step, as ATP would not hydrolyze in the absence of bound GMP.

Another novel experiment in Zocchi's lab was studying GK's mechanical properties through a technique they call nanorheology (Figure 3). In this technique the protein tethers Gold nanoparticles, to a gold surface. The particles are driven by an AC electric field while their displacement is synchronously detected by evanescent wave scattering, yielding the mechanical response function of the protein under study in the frequency domain (Wang and Zocchi, 2010). This technique can be thought of as using a rheometer (Barnes and Hutton, 1989) to measure the deformation of the substance under study, in this case, the protein, in nanometer scale. Analyzing the frequency response of the protein one can determine its dynamics. For GK the results showed that the amplitude of deformation decreases as $1/\omega$ (the inverse of frequency of the applied force) for low frequencies and it plateaus at higher frequencies. Therefore, it was concluded that the protein can be modeled as a viscoelastic material: its mechanics are best described by a viscous fluid at low frequencies of the applied force and by an elastic spring at high frequencies. The frequency beyond which the protein shows elastic behavior is the corner frequency. Another interpretation of this result is that the system is dissipative in low frequencies and non-dissipative in high frequencies. It has been suggested (Alavi *et al.,* 2015) that this internal dissipation can be associated to the dynamics of the hydration layer of the enzyme. It was shown that perturbing the hydration layer of GK through small amounts of chaotropic and kosmotropic agents can have a significant effect on its internal dissipation(Casanova-Morales *et al.,* 2018a). Furthermore, the closed state of the enzyme (GMP bound) was shown to behave dramatically different than the open state in the viscoelastic regime while behaving similarly in the elastic regime (Wang and Zocchi, 2010; Qu *et al.,* 2012). The open state is therefore 'softer' as it can access the soft more dissipative modes easier through the hinge motion which is related to the viscoelastic transition. In this view, the hinge would flow which can be a result of localized melting and refolding as was previously suggested in studies using structure-based coarse-grained simulations (Miyashita *et al.,* 2003; Whitford *et al.,* 2007).

Therefore, the hinge motion can be regarded as a dissipative transition between the extended and compact conformations of the protein. When the enzyme is in its unbound state, the structure between the two conformations can be driven by applying an external oscillatory force. Then in each conformational cycle, the structure hops from one state to another on the energy landscape, this hopping is what is regarded as 'viscoelastic transition' in nanorheology experiments and 'cracking' in molecular dynamic simulations. In this view, the dissipative viscous regime is extended for the unbound state.

However, when the enzyme is in its bound state, the structure is more confined with minimal oscillations around the bound state, that is, smaller hops locally closer to the bound state on the energy landscape. One can think of this 'viscoelastic transition' or 'cracking' as local regions of the protein unfolding during conformational transitions in order to reduce the significant strain in the hinge region.

This viscoelastic model however only describes the behavior of the enzyme versus frequency and not versus force. A non-equilibrium phase diagram was obtained in the frequency force plane separating liner elasticity dynamics from softer viscoelastic dynamics (Qu *et al.,* 2012).

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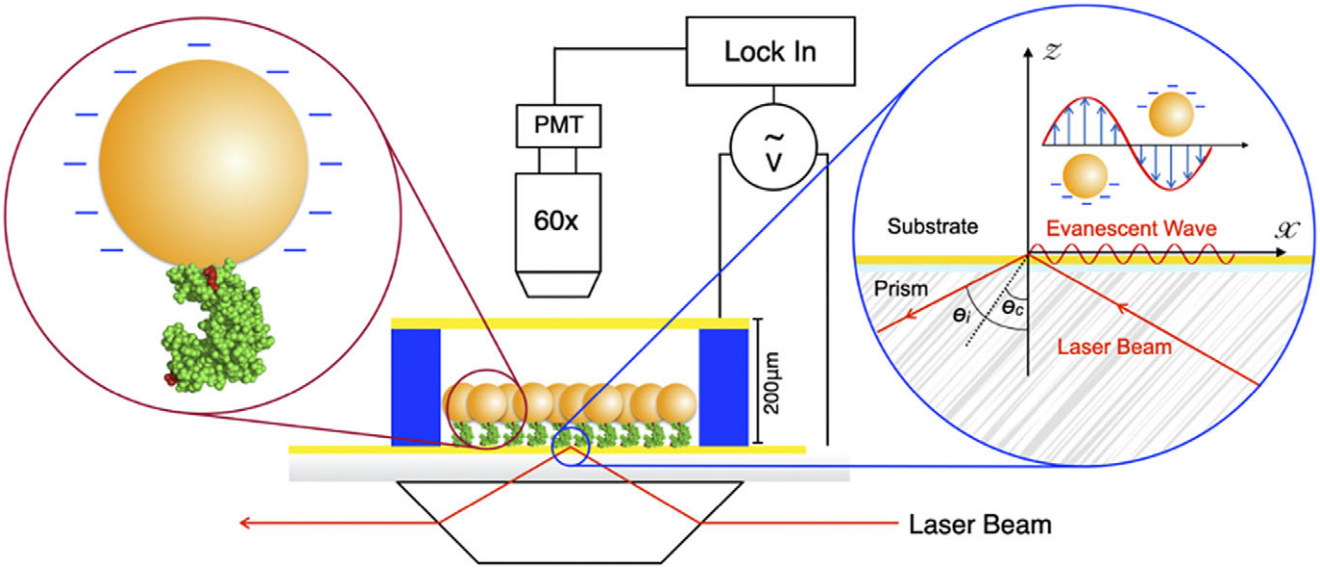

Figure 3. Nanorheology setup, showing the flow chamber with enzyme-tethered GNPs (enzyme shown in green and GNPs shown as golden spheres), the parallel plates capacitor geometry used for mechanical excitation, and the evanescent wave scattering optics used for readout.

Therefore, enzyme's mechanics is non-linear and displays a softening transition as a function of force as seen in experiments where the frequency of the applied force is kept constant while the amplitude of it varies. The critical force at which the transition happens depends on its frequency. Similarly, the corner frequency depends on the amplitude of the applied force. This nonlinearity can also be explained by the concept of barrier crossing in an energy landscape, similar to the problem of escape over a barrier for a particle in thermal bath which suggests local unfolding, namely cracking, can be involved in these conformational motions(Zocchi, 2018). In this view, the corner frequency can be regarded as the rate of breaking the specific bond structure of the ground state confirmation of the molecule, namely the escape rate from a barrier in the energy landscape. This rate increases with the force, that is, ω_c increases as the amplitude of the external force increases. In the same view, linear elasticity regime would extend at higher frequencies: at higher frequencies (on shorter timescales) a bigger force is needed to drive the structure from one regime to another. This nonlinearity is consistent with the idea of local unfolding as a relaxing mechanism in regions with high strain. The rate of this cracking would depend on the amplitude of the applied force and a bigger force is required to have local unfolding in shorter timescales.

BiP: The master regulator of the ER

One of the most important chaperones in the ER is the molecular motor BiP protein (immunoglobulin heavy-chain Binding Protein, around 72 kDa and 8 nm long, Figure 2*c*). BiP (also known as HSPA5 and Grp78), a monomeric ATPase conserved across species, has been referred to as the master regulator of the ER because of the broad and crucial roles that it plays in ER processes and functions (Hendershot, 2004; Alfaro-Valdés *et al.,* 2018), such as protein synthesis, folding, assembly, circadian cycle and as molecular motor in translocation across the ER (Zimmermann *et al.,* 2011; Behnke *et al.,* 2015; Pickard *et al.,* 2019). Although at the molecular level, the study of BiP is still in its early stages, some research groups have published findings of great value. These findings suggest that this protein could be a key player in various fields, such as in the detection and treatment of serious diseases (neurodegenerative diseases, cancer, heart diseases, among others; (Shields *et al.,* 2012; Kosakowska-Cholody *et al.,* 2014; Park *et al.,* 2017; Ichhaporia and Hendershot, 2021). Also, it has been implicated recently in the binding of the coronavirus SARS-CoV-2 spike protein to its membrane receptor (Dores-Silva *et al.,* 2020; Ibrahim *et al.,* 2020; Shin *et al.,* 2021). Structurally, BiP is formed by two domains: a nucleotide-binding domain (NBD) with ATPase activity, connected by a flexible hydrophobic linker to the substrate-binding domain (SBD). The SBD can be further divided into a compact β-sandwich domain harboring a cleft for substrate binding and an α-helical domain at its C-terminal end, the so-called 'lid' (Zhu *et al.,* 1996). Many conformational changes, such as the opening and closing movement of the lid and the variation in the distance between the SBD and NBD, have been associated with the ATPase cycle of BiP in the ER. Once BiP binds K^+ and ATP, its NBD and SBD domains come into close proximity to each other and the lid of the SBD opens, adopting a form that binds substrate proteins with low affinity. Also, several BiP cofactors have been discovered that assist in controlling the substrate-binding cycle and its localization within the ER (Otero *et al.,* 2010; Braakman and Hebert, 2013; Pobre *et al.,* 2019).

Binding of BiP to its substrate

After the Mg^{2+} dependent hydrolysis of ATP, BiP enters a state with low on and off rates for substrate binding/unbinding, respectively (Behnke *et al.,* 2015). For elongated peptide substrates (such as the one that translocate), the lid closes over the bound substrate; for globular substrates, there are direct interactions between the lid and the substrate, although the lid may not be completely closed (Behnke *et al.,* 2015). The SBD and NBD become farther apart upon substrate binding and ATP hydrolysis, being less pronounced for globular substrates. ADP must be exchanged for ATP to release the protein substrate and make BiP available for another round of client binding. Ca2+ increases the affinity for ADP, whereas Nucleotides Exchanges Factors (NEF) such as Grp170 and Sil1 facilitate the nucleotide exchange reaction (Behnke *et al.,* 2015).

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Conformational changes in murine BiP during the ATPase cycle have been determined by Förster Resonance Energy Transfer (FRET) at the single molecule level, showing that NBD and SBD come into close contact with a mean distance of 58 Å–75 Å (Marcinowski *et al.,* 2011). Additionally, by using NMR residual dipolar coupling, spin labeling, and dynamic methods, it has been determined in DnaK (a bacterial Hsp70 protein similar to BiP) that the NBD and the SBD are loosely linked and can move in angles of 35° with respect to each other (Bertelsen *et al.,* 2009). Moreover, the distance between the base and the lid of the SBD domain in Hsp70 has been calculated to be 77 Å by means of FRET (Mapa *et al.,* 2010). Also, a crystal structure of human BiP bound to ATP that shows similar distances has been described (Yang *et al.,* 2015). The conformational changes and movements of BiP are not independent for each domain because an important allosteric communication and coupling exists between them (Marcinowski *et al.,* 2011; Casanova-Morales *et al.,* 2018b). In this study, the authors determined that nucleotide binding resulted in concerted domain movements of BiP (Marcinowski *et al.,* 2011). Conformational transitions of the lid domain allowed BiP to discriminate between peptide and protein substrates. Without single molecule approaches it is very difficult to determine how BiP binds to its substrate, since the substrate of BiP is an unfolded peptide, and if we unfold the substrate, we may also unfold BiP. However, by optical tweezers manipulation, we can specifically unfold the substrate without affecting BiP. We recently developed a method to measure how BiP binds to its substrate using optical tweezers (Ramírez *et al.,* 2017; Rivera *et al.,* 2023a). A tethered protein is pulled and relaxed by its N-and C-terminus to mechanically unfold and refold it, respectively, while recording the force and the trap position and measuring the time during which the protein does not refold after an unfolding event. This allows us to obtain the time that BiP is bound (or the inverse k_{off}) to its substrates while the substrate protein remains unfolded (Ramírez *et al.,* 2017). Also, we developed chimera proteins in which a portion of the protein is unfolded and another is unfolded, that allows to study how BiP works in translocation (Alfaro-Valdés, 2019).

BiP's mechanochemical mechanism

In spite of the crucial roles of BiP during translocation, it is not fully understood whether the action of BiP is through an active mechanism of pulling (as a power stroke), mediated by the binding/hydrolysis of ATP, or as a ratchet mechanism. The hypothesis of the ratchet mechanism is supported by employing antibodies against the polypeptide chains passing through the ER lumen (Matlack *et al.,* 1999) and using a protection assay with substrates that unfold at different forces (Alfaro-Valdés, 2019). Evidence for the translocation mechanism has been obtained using coarse-grained model simulations (Assenza *et al.,* 2015). This study suggests that Hsp70 chaperones use an 'entropic pulling mechanism' applying a force of about 15 pN, and proposes that the Hsp70's would use a combination of ratchet and power stroke mechanisms (De Los Rios *et al.,* 2006). Translocation in all eukaryotes is likely to be similar to yeast, because of the high identity of amino acids between their channels. The channel interacts with the Sec62/Sec63 complex, with BiP acting as a molecular motor to bias the passive movement of a polypeptide in the Sec61 channel. In bacterial post-translational translocation, the channel interacts with the cytoplasmic ATPase SecA. SecA moves polypeptides through the SecY channel to the periplasm by a 'push and slide' mechanism (Bauer *et al.,* 2014). Archaea probably use both co-translational and post-translational translocation, but it is unknown how post-translational translocation occurs because these organisms lack SecA, Sec62/Sec63 complex and BiP (Zimmermann *et al.,* 2011; Park and Rapoport, 2012). Thus, these motor enzymes must couple one or more chemical steps to perform mechanical work. To understand how BiP is doing its work during post-translational translocation, it is necessary to consider that there must be a concerted mechanism engaging mechanical work with changes in the conformational arrangements (or local unfolding) of BiP, and now with single-molecule experiments is it possible establish the correlation between them. A recent paper shows a direct observation of chemo-mechanical coupling in DnaK by optical tweezers (Singh *et al.,* 2022). They observe that the SBD lid closure is strictly coupled to the chemical steps of the ATP hydrolysis cycle showing a clear domain motion dependent on this chemical step.

Viscoelastic behavior of BiP in nanorheology

The previously described nanorheology technique allows for studying the mechanical behavior of the protein in bulk and obtaining physical properties of the protein (stiffness and elasticity) which could be explained by local unfolding.

As observed for GK, BiP protein in the folded state behaves like a viscoelastic material. The protein becomes softer when bound to nucleotides ADP and ATP. In the presence of ADP, the linker separating the SBD and NBD domains is elongated which leads to a significant decrease in rigidity. In the presence of ATP, the lid is more flexible and the domains are closer (Bertelsen *et al.,* 2009; Mapa *et al.,* 2010; Casanova-Morales *et al.,* 2018b; Yokoyama *et al.,* 2022). However, BiP becomes more rigid when bound to the HTFPAVL peptide substrate because the lid of BiP is closed, generating a compact and thus rigid state.

Furthermore, peptide binding was reported to dramatically increase the affinity for ADP (Casanova-Morales *et al.,* 2018b). This shows that the connection between the SBD and NBD domains presents an allosteric coupling (Swain *et al.,* 2007; Chakafana *et al.,* 2019).

Nanorheology versus optical tweezers: Complimentary techniques to study local unfolding

Nanorheology and optical tweezers are complementary techniques because they operate in different force and frequency regimes. Usually in optical tweezers (for a detailed description of this instrument please see(Smith *et al.,* 2003; Bustamante *et al.,* 2014; Sánchez *et al.,* 2022) the force versus extension plot starts around a few picoNewtons to 67 piconewtons and in nanorheology the force is estimated to be in the low piconewton regime (Qu and Zocchi, 2013). A typical tweezers pulling is around 100 nm/sec and the stiffness is 0.1 pN/nm, then the complete pulling (if you pull up to 30 pN for example) will take around 6 sec, meaning 0.1 Hz. The force versus extension plot obtained by optical tweezer can be fit to the worm-like chain interpolation formula (Bustamante *et al.,* 1994) which does not change much when the speed of pulling is changed. Interestingly the WLC fitting works well for dsDNA in the whole range of forces, but in the case of proteins the force versus extension plot deviates from the WLC fit at low forces (Bechtluft *et al.,* 2007; Kaiser *et al.,* 2011; Wilson, 2011; Bianco *et al.,* 2015; He *et al.,* 2019; Li *et al.,* 2021), probably due to localized unfolding/folding. Polypeptides may exhibit non-WLC behavior at lower forces if localized structures form during relaxation, such as beads on a string caused by hydrophobic collapse, or off-pathway folding intermediates which delay the final state.

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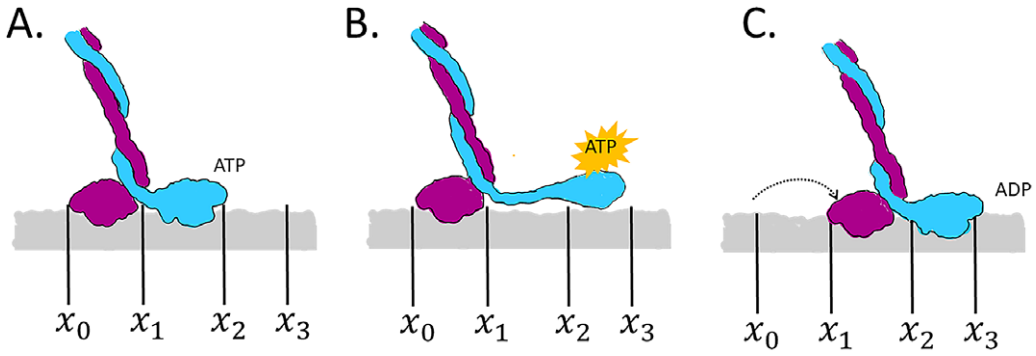

Figure 4. Kinesin1 as molecular machine. (*a*) Kinesin1 is in initial position x0–x2. (*b*) After ATP hydrolysis a partial unfolding of one of its dimers allows it to advance to the x3 position. (*c*) Finally, the folding of the dimer drags the protein to a final position x1–x3. Kinesin1 use ATP hydrolysis as a source of energy to move along a track.

Complementary, in Nanorheology where the frequency of the applied force is 10–200 Hz, a viscoelastic behavior is seen at lower forces which may correspond to local unfolding and help explain the deviation in the force versus extension plot obtained in optical tweezers. Currently, there is no comprehensive model for this viscoelastic transition (Zocchi, 2018). Developing such a model would be important for a complete description of protein's behavior under force.

Conclusion

In the framework described here, the enzyme is viewed as a cyclic engine taking many different conformations throughout the cycle. These conformational states produce a 'rocky' energy landscape as seen in Figure 1. The difference in the chemical potential of substrates and products drives the cycle in one direction. The cycle is initiated when the substrate binds. The force exerted by the substrate on the different parts of the enzyme then drives the 'open to closed' conformation leaving the protein in the closed stressed state. This step can be thought of as the substrate pulling the structure towards the closed conformation with a constant force which results in an elastic deformation of the molecule followed by a larger viscous deformation (a.k.a flow). In the energy landscape picture, this step can be interpreted as the jump between the two conformations, a flight from one region to another in Figure 1. This jump can be due to local unfolding as a mechanism to reduce strain that resulted from the force between the substrate and the enzyme. While in the closed state, internal stresses might change due to the change in the force between the substrates and the protein, however, the overall conformational change is very small (Delalande *et al.*, 2011). In the energy landscape, these small conformational changes are regarded as confined diffusion within one region. The next step in the cycle involves the reaction. During this step, significant strain energy is built which could be released by local unfolding/refolding. The products are then released and the internal restoring force of the enzyme's structure brings it back to initial open state. Typically for motor proteins, this cycle is fast-slow: the time it takes the structure to close is shorter than the time it takes the structure to open (Zocchi, 2018).

The chronology above is considering only one substrate for simplicity and in general, is only an approximate model. The real system consists of ~10^4 atoms and thus moves in a phase space consisting of the coordinates and the momenta of all the atoms. This model however provides a qualitative representation for the ensemble-averaged trajectory of the enzyme + substrate system during the enzymatic cycle. Simply put, throughout the cycle the strength of local interactions change as the enzyme deforms, and therefore different regions of the molecule could locally unfold (for exemplification of the cycle see kinesin in Figure 4).

Considering the gap of information in the understanding of the role of forces in the mechanical–structural processes involved in enzyme catalysis, and that the 'strain-induced' idea remains somewhat hypothetical, studying the forces at a single molecule level could provide a novel and feasible approach. The *in singulo* studies carried out by optical tweezers, coupled or not to fluorescence and nanorheology, could be used to determine the energies that govern these processes. In this context, questions such as the following can be now answered: Is AK catalysis an example of the strain-induced theory? Does GK undergo a cracking mechanism? How does the applied force affect the turnover of AK, GK, and BiP? At what levels does the applied force help or hinder catalysis, and what forces will prohibit the enzyme from binding the substrate or achieving catalytic turnover? Is BiP unfolding correlated with the translocation step and speed?

This approach could provide valuable insights into the underlying mechanisms of enzyme catalysis and help to shed light on the role of forces in these processes.

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Competing interest. All authors have no competing interests to declare.

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