Watching the walk: observing chemo-mechanical coupling in a processive myosin motor

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Molecular motors are cellular nanomachines that convert the energy from nucleotide binding, hydrolysis, and product release into mechanical work. Because molecular motors contribute to fundamental processes in all living organisms, including genome replication, gene transcription, protein synthesis, organelle transport, and cell division, understanding how the chemical (ATP utilization) and mechanical (motility) cycles are linked is of fundamental importance. A recent study reports the direct visualization of simultaneous nucleotide binding and mechanical displacement of a single myosin 5a molecule, a processive molecular motor protein that takes successive ~36-nm steps along actin filaments of the cytoskeleton. This new work demonstrates an exciting advance in single-molecule enzymology and advances our understanding of the link between chemical catalysis and mechanical work in molecular motors, particularly those that operate under internal and external loads.
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Numerous key biological processes in eukaryotic cells require the rapid transport of intracellular components to specific locations. Long-range intracellular transport occurs along microtubules and actin filaments, self-associating polymers of the cytoskeleton, which provide the cell shape and mechanical integrity, and serve as molecular superhighways and roads along which molecular motor proteins translocate. Cytoskeletal motor proteins mediate transport processes by converting the energy from ATP binding and hydrolysis into unidirectional, mechanical stepping along microtubules or actin filaments.

Myosins comprise a diverse family of molecular motor enzymes that perform mechanical work along actin filaments. The family is characterized by a conserved catalytic motor domain (referred to as "head") that binds to actin, hydrolyzes ATP, and performs mechanical work. ATP binding, hydrolysis, and product release cycle myosin through a series of conformational states with weak and strong actin binding affinities.

In Figs. 1 and 2, molecular models of the actomyosin complex and ATPase cycle intermediates were manipulated using MacPyMOL (available at http://www.pymol.org). The converter domain of *Dictyostelium* myosin 2 in the pre-power stroke state ($ADP.P_i$ state; PDB 1VOM) was aligned with the post-power stroke structure of chicken myosin 5a (PDB 1OE9). The actomyosin complex from a model of nucleotide-free heads in insect flight muscle was used (PDB 1M8Q). An atomic model of two-headed myosin 5a in its inhibited state was adapted for use in Fig. 2 (PDB 2DFS).

Mechanical displacement does not result from a tilting of the head relative to the actin filament axis but rather from the rotation of a "lever arm" that extends from the motor domain and amplifies the conformational changes at the catalytic site associated with ATP binding, hydrolysis and product release. Rotation of the lever arm during strong attachment to actin is referred to as the "power stroke," and results in a mechanical displacement. Force generation and work output (mo-

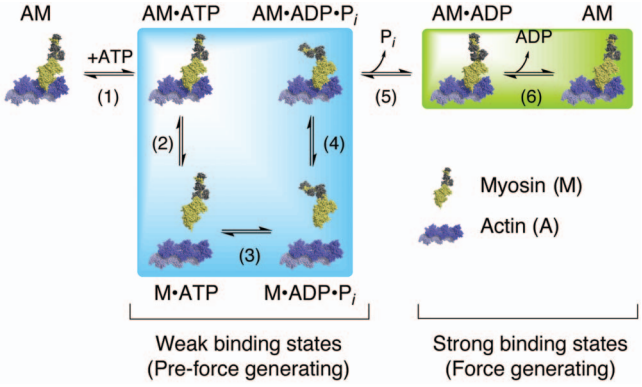

Figure 1. The actin-activated myosin ATPase cycle. Binding and hydrolysis of ATP cycle myosin through weak (blue box) and strong (green box) actin binding states. Upon ATP binding (1), myosin rapidly dissociates from actin (2); a conformational change, referred to as the "recovery stroke," enables myosin to rapidly hydrolyze ATP and form the products ADP and P_i (3). Although myosin bound to ADP and P_i is an unstable, high energy intermediate, it is kinetically very stable, releasing P_i very slowly in the absence of actin (less than once every 10 s). Actin binding (4) accelerates P_i release (5) from myosin·ADP·P_i, an event tightly coupled to reversal of the recovery stroke; this "power stroke" results in a mechanical displacement (and force production) as myosin relaxes and rotates the lever arm back to the post-power stroke position. Myosin subsequently releases bound ADP (6) and repeats the cycle with the binding of another ATP to the empty nucleotide-binding pocket.

tility) are coupled to inorganic phosphate (P_i) release although it is still not known with absolute certainty if P_i is released before or after lever arm movement. It is generally believed that the chemical (ATPase) and mechanical (motility) cycles are tightly coupled and that utilization of a single ATP molecule corresponds to a single mechanical displacement along an actin filament. However, experimental data has argued in favor of a loose coupling mechanism in muscle myosin (Ishijima *et al.*, 1998) and in efficient transport by ensembles of processive kinesin motors (Bieling *et al.*, 2008).

Although all myosin classes share similar structural and ATPase cycle mechanisms, modulation of the ATPase cycle rate and equilibrium constants confers specific properties to the motors that enable them to produce diverse types of motility (De La Cruz and Ostap, 2004). Such enzymatic tuning is best explained in terms of the "duty ratio," or the fraction of ATPase cycle time that myosin spends attached to actin filaments and performs work (Howard, 2001). A low duty ratio (<0.5) motor spends less than half its ATPase cycle time attached to its track. Functionally, this adaptation allows for transient (and rapid) force production along actin but requires an assembly of many motors for continuous movement, such as occurs for skeletal muscle myosin. Conversely, a high duty ratio (>0.5) allows for longer actin attachment times and continuous force production at low motor densities. For example, muscle myosin has adapted for short, rapid contractions and it tugs intermittently on actin filaments. Muscle myosin has a low duty ratio and remains dissociated from actin much of the time so multiple myosins are needed to sustain constant movement. In contrast, the high duty ratio motor, myosin 5a (as well as myosin 6), are processive and individual two-headed molecules take multiple ~36-nm steps along an actin filament for long distances without detaching.

To sustain processive movement as an individual molecule, at least one head of a processive two-headed myosin must be attached to actin at all times or else random thermal forces could cause it to diffuse away from its track. Each myosin 5a motor domain has a high duty ratio and remains attached to actin for most of its ATPase cycle because of a slow, rate-limiting ADP release rate, which limits detachment from actin in the presence of ATP (De La Cruz *et al.*, 1999; De La Cruz *et al.*, 2000), thereby allowing the native two-headed myosin 5a molecule to keep at least one head attached to actin at all times. Interactions with the ADP-associated magnesium ion (Hannemann *et al.*, 2005) and distinct thermodynamic components (Robblee *et al.*, 2005) contribute to the slow release and strong ADP binding affinity of actomyosin 5a relative to that of muscle actomyosin.

Although not absolutely required to achieve processivity, allosteric head-head communication could increase the number of steps taken by myosin 5a (i.e., processive run length) and ensure efficient delivery of cargo. Single-molecule detection of fluorescent motors reveals that myosin 5a walks along actin following a "hand-over-hand" mechanism in which the two heads alternate leading and trailing positions along the actin filament (Forkey *et al.*, 2003; Yildiz *et al.*, 2003). Because ADP keeps myosin strongly bound to actin and ADP release is rate-limiting, head-head coordination could arise if the pushing and pulling forces exerted by the two heads when bound to actin modulated this critical step.

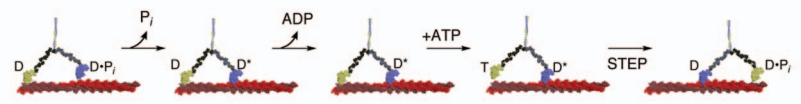

Figure 2. Predominant myosin 5a stepping pathway. Myosin 5a predominantly walks through a pathway in which the main steady-state intermediate populated binds actin and ADP with both heads. The lead head releases P_i rapidly but cannot undergo its powerstroke. ADP release from this state, designated "D*," occurs slowly while ADP release from the rear head occurs at the unconstrained rate of ~15 s^{-1}. Two-headed detachment (and run termination) most likely occurs if strong actin attachment and P_i release by the lead head is slower than ATP induced actin detachment of the rear head.

ADP release has been shown to be force-dependent in many myosin classes (Laakso *et al.*, 2008; Nyitrai and Geeves, 2004), including myosin 5a (Oguchi *et al.*, 2008; Purcell *et al.*, 2005; Veigel *et al.*, 2005) indicating that this is a reasonable possibility for allosteric communication and gating. Transient kinetic (Olivares *et al.*, 2006; Rosenfeld and Sweeney, 2004) and isotopic exchange (Olivares *et al.*, 2006) solution studies as well as single-molecule imaging and nanometry studies of individual myosin 5a motor domains (Oguchi *et al.*, 2008; Purcell *et al.*, 2005; Veigel *et al.*, 2005) or processively translocating dimers (Baker *et al.*, 2004; Dunn and Spudich, 2007; Kad *et al.*, 2008; Uemura *et al.*, 2004) indicate that allosteric head-head coordination exists, though directly correlating nucleotide binding events to mechanical stepping events has not been achieved. Therefore, the ability to simultaneously observe nucleotide binding and mechanical stepping would serve to provide important insight into how myosin 5a steps along actin.

SIMULTANEOUS OBSERVATION OF NUCLEOTIDE BINDING AND STEPPING

Sakamoto and colleagues (2008) have succeeded in confirming the tight chemo-mechanical coupling during processive myosin 5a stepping along actin. This new work highlights two important developments in studying myosin processivity. One involves the precise tracking of a single fluorescent molecule by fitting fluorescence intensity to a two-dimensional Gaussian function. This method of fluorescence imaging with 1-nm accuracy (FIONA) allowed for the demonstration of hand-over-hand movement of myosin 5a (Yildiz *et al.*, 2003) and kinesin (Yildiz *et al.*, 2004). The other development is the use of a novel fluorescent nucleotide analog, deac-aminoATP. This analog, first synthesized by Webb and colleagues (Webb *et al.*, 2004) and characterized for use with myosin 5a (Forgacs *et al.*, 2006) has the very useful property of ~25-fold fluorescence increase upon binding to myosin and actomyosin in solution and a ~4-fold increase in single-molecule assays. This property yields higher signal-to-noise ratios at higher nucleotide analog concentrations compared to analogs previously used in single-molecule studies such as Cy3-ATP, which is limited to a concentration of ~100 nM because of a <1.5-fold fluorescence increase upon binding myosin (Komori *et al.*, 2009). Another important feature of deac-aminoATP is that it has slow ADP release kinetics (~1 s^{-1} for deac-aminoATP versus ~15 s^{-1} for ADP and mantADP). This is particularly important for measurements using FIONA, which relies on collecting a sufficient number of photons during each step for precise tracking and analysis.

Using combined FIONA on myosin 5a labeled with a fluorescent probe attached to calmodulin and deac-aminoATP, Sakamoto and colleagues directly observe that ADP release occurs preferentially from the rear head and that ATP binding to the rear head causes a forward step. Interestingly, the observed rate of ADP release is not accelerated indicating that intramolecular forces generated when both heads bind actin do not affect ADP release from the rear head during processive stepping (Forgacs *et al.*, 2008; Oguchi *et al.*, 2008; Olivares *et al.*, 2006; Purcell *et al.*, 2005; Rief *et al.*, 2000; Rosenfeld and Sweeney, 2004). Rather, their findings demonstrate that gating during processive stepping occurs in the lead head as interpreted from kinetic studies in bulk (Rosenfeld and Sweeney, 2004) or nanometry measurements of isolated motor domains (Oguchi *et al.*, 2008; Purcell *et al.*, 2005), such that rearward loads exerted by the attached trailing head slow nucleotide dissociation from the lead head. The chemical state of the lead head bound nucleotide is not known with absolute certainty. However, kinetic studies indicate that ATP hydrolysis and Pi release are rapid (De La Cruz *et al.*, 1999; Rosenfeld and Sweeney, 2004), thereby favoring a processive stepping mechanism in which the rate constant for release of bound ADP from the lead head is slowed (Figure 2). Analogous behavior has been observed for class I myosins, for which ADP release is reduced ⩾75-fold under low external loads (Laakso *et al.*, 2008).

CONCLUSIONS

The ability to observe both the chemical and mechanical state of a dimeric motor protein has been somewhat of a holy grail in single-molecule biophysics. It requires the correct conditions and reagents in order to gain anything useful. In the case of myosin 5a, Sakamoto and colleagues have applied several technologies including total internal reflection microscopy and fluorescent nucleotide analogs to successfully dissect nuances of the mechanism of processive stepping along actin. Recently, an independent study also demonstrated the successful use of Cy3-ATP analogs and optical trapping to study tight coupling during single encounters of a single motor domain of myosin 5a (Komori *et al.*, 2009). Such combined application of single-molecule detection technologies brings some hope to the study of other motor proteins such as kinesin-1 and myosin 6, whose hand-over-hand movement has been studied using FIONA and optical trapping.

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