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X

ON THE WALKING MECHANISM OF LINEAR MOLECULAR MOTORS

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1. WALKING LIKE A HUMAN?

Many of linear molecular motors, such as myosins and kinesins, have two 'feet' (traditionally called 'heads' or 'motor domains') that bind to a motor-specific track and that each host a catalytic site for hydrolyzing ATP to power unidirectional movement along the track (Kinosita et al., 1998, 2005; Vale and Milligan, 2000; Mehta, 2001; Vale, 2003; Schliwa and Woehlke, 2003; Endow and Barker, 2003; Sablin and Fletterick, 2004). Some of the linear motors, such as conventional kinesin (Brady, 1985; Vale et al., 1985; Howard et al., 1989; Block et al., 1990; Svoboda et al., 1993), myosin V (Cheney et al., 1993; Mehta et al., 1999; Sakamoto et al., 2000), myosin VI (Kellerman and Miller, 1992; Wells et al., 1999; Rock et al., 2001; Nishikawa et al., 2002), and plant myosin XI (Tominaga et al., 2003), are processive, in that a single motor molecule proceeds along a filamentous track for many ATPase cycles without detaching from the track. That the two feet never detach simultaneously from the track (or the ground in case of a human) is an important feature of 'walking,' as opposed to 'running' (Kinosita et al., 1998). In addition, at least for myosin V and conventional kinesin which are known to be processive, convincing evidence exists that these motors throw their two feet forward alternately in a hand-over-hand fashion (Yildiz et al., 2003, 2004; Kaseda et al., 2003; Asbury et al., 2003; Warshaw et al., 2005), just as a human does. In myosin V which has two long and stiff legs (we refer to as a 'leg' the light-chain binding domain and associated light chains of myosin V; see Fig. 1; in the literature this part is often referred to as a 'lever arm'), fluorescence polarization measurements have indicated that each leg changes its orientation every time the myosin steps (Forkey et al., 2003),

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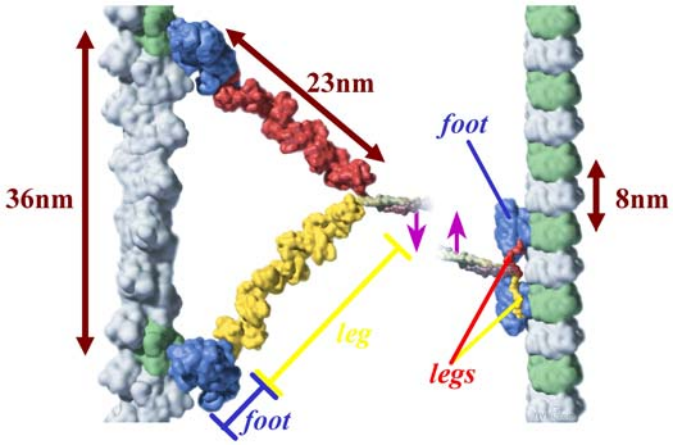

Figure 1. Structural models of myosin V (left) and conventional kinesin (right), adapted from Vale and Milligan (2000). Purple arrows show the direction of motor movement. Left, two feet (heads) of myosin V are shown in blue, the lead leg (light chain binding domain to which six calmodulin light chains are bound) in yellow, and the trail leg in red. The two legs merge with each other to form a coiled coil of α helices (colored gray). Each foot is bound primarily to an actin monomer in green, the front one being the thirteenth monomer from the rear one on the right-handed double helix of filamentous actin (gray). Right, two feet of conventional kinesin are shown in blue, the lead leg and trail leg in red and yellow, respectively. Kinesin's two legs also merge into a coiled coil (colored gray). Each foot is bound primarily to a β tubulin (light green; α tubulin in gray) in a protofilament of a microtubule.

consistent with man-like walking. Recently we have succeeded in attaching a microtubule, as a micron-sized marker, to a leg of myosin V; we observed the microtubule to lean forward and backward on an actin filament as the motor stepped along the filament with an average step size of ~36 nm (Shiroguchi and Kinosita, 2005). All these observations indicate that two-foot processive motors do 'walk,' in that there are moments when both feet are landed on the track, one in front of the other, and that subsequent movements are lifting of the rear foot followed by landing on a position ahead of the pivoting foot. To what extent, then, does the walking mechanism of molecular motors resemble that of a human?

When a man walks, he relies heavily on the mass. Thus, he will allow gravity to pull his lifted foot down, instead of pushing it with his muscles. The foot that has come down will be carried forward by inertia, without requesting much help from muscles. If you attach a big balloon at his head to watch, from far above, how he moves forward, as done in a single-molecule assay, the balloon would move steadily forward with little sign of discrete stepping, because a human being normally exploits the inertia of the body to make movement as smooth as possible. Last but not least, a man does not have to cling to the ground to stay on, except possibly in a typhoon.

For biological molecules in an aqueous environment, mass is virtually absent, whether gravitational or inertial. A protein molecule never sinks toward the bottom of a tube, unless spun in an ultracentrifuge for long hours. This is because the mass is so small and the Brownian motion, or bombardment by surrounding water molecules, is so vigorous. A processive molecular motor must cling tenaciously to its track; otherwise the motor is immediately blown away by Brownian motion. Inertia is also negligible. In the absence of an external force, a protein molecule stops moving in a unique direction

in a matter of 10^{-12} seconds, or within 0.1 nm, and begins to undergo a completely random Brownian fluctuations. This is due to the enormous viscous friction exerted by the aqueous environment, compared to the inertia (Purcell, 1976). If you observe a molecule moving steadily in one direction, it must be pushed (or pulled) all the way by a force.

Another critical difference between a two-foot molecular motor and human being is that a molecular motor does not have a right and a left foot. The two feet, and the two legs, are completely identical. An expected consequence is that the motor would adopt precisely the same posture every time it steps, not every two steps as in human walking. A prediction that follows is that the motor body would rotate by 180° every time the motor steps (Howard, 1996), but this has not been observed so far (Hua et al., 2002; Ali et al., 2002, 2004).

The two identical legs of a molecular motor are joined at their base in twofold symmetry to form a coiled coil of α helices (Kozielski et al., 1997; Li et al., 2003; see Fig. 1). If one foot points to the west, the other foot would point to the east. When the two feet bind to the track simultaneously, though, the two must be oriented in the same direction dictated by the structure of the binding sites on the track. Pointing both toes to a same direction is a natural posture for a human, but, for a molecular motor, doing so would require awkward bending and/or twisting of its legs (Kinosita et al., 2005). The legs thus must be flexible, yet they have to produce forward movement against a backward load.

2. HOW MOLECULAR MOTORS MAY WALK

2.1. Free Joints in the Legs

Orienting both toes forward is easy if there is a free joint(s) in the legs. For muscle myosin (myosin II), optical anisotropy decay measurements have revealed the existence of a free joint at the leg-hip junction, as shown in Fig. 2 (Kinosita et al., 1984; Ishiwata et al., 1987). Electron micrographs of myosin V have indicated that this myosin also has a free joint at the leg-hip junction (Walker et al., 2000). The junction is the point at which the coiled coil of α helices branches into two individual helices (Fig. 1), and thus melting of just one turn of the helix suffices to form a free joint, even though the rest of the helix is reinforced by light chains. In myosin VI, an upper part of the leg, unprotected by light chains, is flexible over ~80 amino-acid residues (Rock et al., 2005). In kinesin which lacks light chains that cover the legs, the entire portion of the legs (called ‘neck linkers’ in the literature; see Fig. 1) is likely flexible. In the original crystal structure (Kull et al., 1996), the leg portion of kinesin was disordered.

With the free joints, taking a standing posture with both feet on the track and both pointing forward will not result in an energy-consuming strain. Also, a free joint(s) in each leg implies that the body (the coiled coil portion) has a considerable freedom of rotation in the standing posture, around the axis of the body. A 180° rotation of the body in either direction can be achieved simply by crossing the two legs below the free joints. This would explain the above-mentioned failure in observing 180° rotations in processive motors: a slight hindrance against the rotation, likely introduced by the attachment of a probe for the observation, would suffice to induce leg crossing to counter

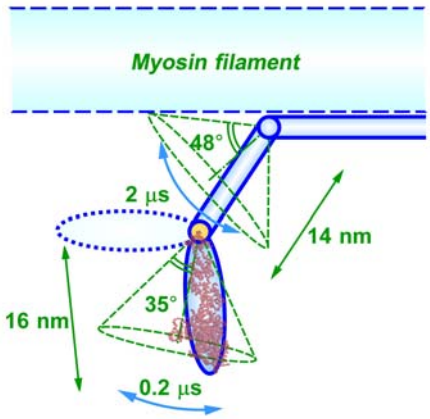

Figure 2. Rotational Brownian motion of muscle myosin (myosin II) on a myosin filament. In a muscle, many myosin molecules are bundled parallel to each other to form myosin filaments, from which myosin feet (and legs) protrude into surrounding medium. Rotational Brownian motion of the feet was assessed by attaching a dye molecule to the foot and measuring the absorption or phosphorescence anisotropy decay after pulsed and polarized excitation of the dye (Kinosita et al., 1984; Ishiwata et al., 1987). The motion could be modeled as wobbling of a prolate ellipsoid of size 16 nm in a cone of semiangle 35°, which in turn wobbles in another cone of semiangle 48°. The size of the prolate matches that of a foot-leg portion of myosin II, as shown in pink in the figure (the structure shown is that of scallop myosin binding MgADP·VO_4, from Houdusse et al., 2000). The result suggests that the two legs of myosin II are connected to the coiled coil (rods shown in blue solid lines in the figure) through a free joint (yellow).

the rotation (Kinosita et al., 2005).

With the free joints, then, does the motor gait appear similar to a human's except for the possible leg crossing? A free joint, made of single bonds between amino-acid residues, will allow rotation virtually in all directions unless hindered by steric constraints. Thus, a leg, once lifted, may go forward through many routes, passing the other leg on the left or right side, or even from above as has been suggested in the electron micrographs of myosin V (Walker et al., 2000). The motion of the lifted leg must be a Brownian one, seeking all possible routes randomly and changing the direction continually. The lifted leg will move forward and backward basically with equal probabilities.

2.2. How to Move Forward

The hand-over-hand mechanism implies that a processive motor alternates between two-foot and one-foot bindings to the track, as has been demonstrated, for example, for kinesin (Kawaguchi and Ishiwata, 2001). To move forward and not backward, a molecular motor, without a brain, must make a correct choice mechanically at least twice in a cycle. Thus, when both feet are on the track, the motor must choose the correct foot (trail foot) to bring up. Then, the lifted foot must land on a forward, not backward, binding site. The prevailing theory for the choices is that a strain-dependent mechanism warrants the lifting of the trail foot and a lever action in the landed foot biases the motion of the lifted foot forward to assure forward landing.

2.3. Which Foot to Bring Up

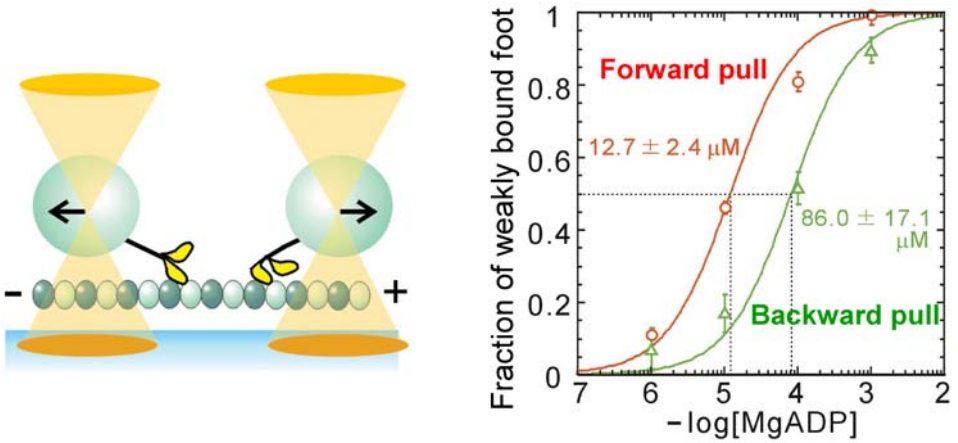

Figure 3. Strain-dependent detachment of kinesin from a microtubule, adapted from Uemura and Ishiwata (2003). A single kinesin molecule was attached to a plastic bead and the bead was pulled along a microtubule with optical tweezers at a constant speed. The kinesin repeatedly attached to and detached from the microtubule, and the force required for each unbinding event, presumably involving only one of the two feet under the experimental conditions employed, was estimated from displacement of the bead from the trap center. The unbinding force could be classified into a weak and a strong class, and the fraction of weakly bound events is plotted in the graph on the right, as a function of the MgADP concentration in the medium. When a foot binds MgADP, its affinity for a microtubule is expected to be low, compared to a foot without a nucleotide (or a foot binding ATP which was absent in this experiment).

When both feet of a processive molecular motor are landed on the track during normal walking, the chemical states of the two feet, whether they bind ATP, ADP plus phosphate, ADP, phosphate, or none, may well be different. Feet in different chemical states are expected to show different affinities for a landing site on the track. Thus, a motor could in principle rely on the sequence of hydrolysis reaction to choose the correct foot to lift, the foot with a weak affinity, because the reaction phase on the trail foot is expected to be ahead of the phase on the lead foot. However, chemical reactions are stochastic and basically reversible. A choice relying solely on the simple phase difference cannot be robust: occasionally the two feet may fall into a same chemical state.

To warrant correct choice, or to insure a proper phase difference between the chemical reactions in the two feet, a motor probably exploits the difference in strain in the two feet (Hancock and Howard, 1999; Mehta, 2001). Because the two feet are connected to each other through the legs, the trail foot is pulled forward while the lead foot is pulled backward in the two-foot landing posture. The difference in the strain could modulate the affinity of the foot for the track, or the preference of the foot for a particular chemical state.

The expected strain dependence has recently been demonstrated in a persuasive experiment by Uemura and Ishiwata (2003). They pulled kinesin along a microtubule either forward or backward, and examined whether detachment of a bound foot required a weak or a strong force (Fig. 3). Kinesin's affinity for a microtubule is known to be weak when it binds ADP (Hackney, 1994; Uemura et al., 2002). Under the physiological MgADP concentration of ~10^{-4} M, Uemura and Ishiwata found that a weak

forward pull was sufficient to detach a bound foot from a microtubule, whereas a backward pull often had to be strong to induce detachment (Fig. 3). Thus, from a two-foot landing state, a trail foot under a forward strain will be readily detached while a lead foot remains bound.

The ADP dependence of the strain effect (Fig. 3, right) is revealing: a forward pull increases the affinity of a kinesin foot for ADP (dissociation constant 13 μM), whereas a backward pull decreases the affinity (dissociation constant 86 μM). The interpretation is as follows. Binding of ADP to a kinesin foot induces a conformational change of the foot such that its affinity for a microtubule decreases. By the law of action and reaction, then, inducing that conformational change by an external (or internal) agent should increase the affinity of the foot for ADP. A forward pull is just such an agent. A forward pull tends to change the conformation of the foot into the one that shows a low affinity for a microtubule and that also shows a high affinity for ADP (let us call this a lifting conformation). A backward pull, on the other hand, tends to stabilize the conformation with a high affinity for a microtubule and low affinity for ADP. A similar argument applies to F_1-ATPase, where ATP binding induces counterclockwise rotation and counterclockwise rotation increases the affinity for ATP (Yasuda et al., 2001; Kinosita et al., 2004).

Kinetically, two scenarios are possible: pulling a kinesin foot forward induces ADP binding, halfway toward the lifting conformation, and the ADP binding accelerates the conformational change to final foot lifting; or, a forward pull may lift a foot before it binds ADP and then the lifted foot binds ADP, which will prevent re-landing. Either way, the end result is a foot that binds ADP and that is dissociated from the microtubule. The choice between the two scenarios must be a stochastic one. Either may predominate over the other, but both must take place in the long run. In the world of molecules working with energies of the order of thermal energy, k_BT, where k_B is the Boltzmann constant and T the absolute temperature (e.g., a tenfold difference in affinity corresponds to a free-energy difference of 2.3 k_BT), anything can happen, albeit rarely (a process requiring 10 k_BT would take place, in the absence of an external energy supply, once in exp(10) ~ 20,000 trials, on the average).

Note that the argument above also applies to normal walking where the ADP state is reached by phosphate release from the ADP+phosphate state. Release of phosphate tends to induce the lifting conformation, and a forward pull promotes the phosphate release. By the law of action and reaction, a backward pull prevents the release of phosphate, keeping the foot landed.

Recently, strain-dependent detachment of a single foot of mouse myosin V from actin has directly been observed in a construct composed of a single foot connected to a single leg carrying six light-chain binding motifs (Veigel et al., 2005). A forward pull at 1.4 pN at 3 μM ATP reduced the lifetime of attachment from 390 ms to 170 ms, whereas a backward pull at 2.4 pN increased the lifetime to 733 ms. Similar, but somewhat different, results have been reported for single leg-foot constructs of chicken myosin V (Purcell et al., 2005). For the construct carrying six light-chain binding motifs, a forward pull at ~2 pN at 1000 μM ATP resulted in dissociation from actin at the rate of 15 s^{-1}, which was close to the rate at no load. A backward pull at ~2 pN, on the other hand, significantly reduced the dissociation rate to 1.5 s^{-1}. Results at 10 μM ATP were not grossly different. In the case of myosin (V), detachment of a foot is considered to result from the release of ADP followed by ATP binding (De la Cruz et al., 1999; Reif et

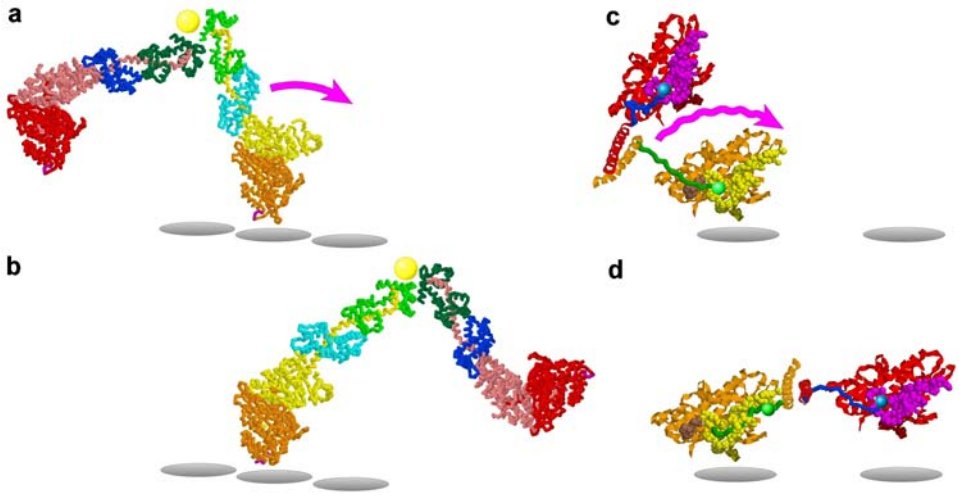

Figure 4. Lever action in two-foot motors which, in this figure, move toward right. (**a, b**) Scallop myosin. This myosin is non-processive, but its structure has been solved up to the leg portion. Postulated lever action is shown by an arrow: the dark-yellow leg (the long α-helix) rotates between *a* and *b*. Flexible joints likely exist around the location indicated by the yellow ball, beyond which the two α-helices form a coiled coil (not shown in the figure). Thus, the pink leg presumably undergoes rotational diffusion around the yellow ball. The lever action of the dark-yellow leg biases the Brownian motion of the red foot forward. Each figure is composed of two structures of scallop myosin subfragment 1 (S1), arranged arbitrarily. The orange foot/dark-yellow leg in *a* represents the structure of S1 binding MgADP·VO_4, and that in *b* is S1 without a nucleotide (Houdusse et al., 2000). The red foot/pink leg is S1 binding MgADP, which is supposed to mimic a structure immediately after detachment from actin (Houdusse et al., 1999). Cyan/blue, essential light chains; green/dark-green, regulatory light chains. The gray disks with a diameter of 5.5 nm represent myosin-binding sites on an actin filament. (**c, d**) Conventional kinesin. The blue and green legs ('neck linkers') between the balls are presumably flexible, and thus the red foot undergoes Brownian motion. The flexible green leg in *c* 'docks' onto the dark-yellow portion of the orange foot, in an ATP-dependent process, through Brownian motion (wavy arrow). After docking (*d*), the Brownian motion of the red foot occurs around the green ball, and thus is biased toward the forward binding site. The docking is equivalent with the lever action in myosin. The docking site (magenta) in the red foot is yet to bind the blue leg (after landing). The gray disks with a diameter of 4 nm represent kinesin-binding sites on a microtubule. The figures are constructed from a structure of a dimeric kinesin (Kozielski et al., 1997) by assigning arbitrary structures to the neck linkers (except the green one in *d*) and orienting the central coiled coil (stalk) arbitrarily.

al., 2000). Thus, a forward pull presumably changes the conformation of a landed foot to one having a low affinity for ADP, and a backward pull stabilizes the conformation having a high affinity for ADP.

2.4. Which Way to Land: Lever Action and Biased Diffusion

After the proper, trail foot is lifted, the next problem is to let the lifted foot choose a correct site for landing. With a free joint(s) in the legs, no force can operate on the lifted foot to move it forward. The foot can move only by Brownian motion, which carries the foot in either direction, with an equal probability unless biased. Yet the foot must land on a forward, not backward, site.

A mechanism that is widely accepted, at least for myosin (V), is the lever action, or an ankle action in the landed foot. A chemical reaction in the landed foot of myosin,

presumably phosphate release, induces forward bending of the ankle, resulting in leaning of the stiff leg forward (Fig. 4). This lever action, originally proposed for myosin II (Huxley, 1969; Huxley and Simmons, 1971), moves the hip portion forward. The Brownian motion of the lifted leg is thus biased forward, because the pivot(s) for the random rotational Brownian motion (yellow ball in Fig. 4) has now been displaced forward by the active (= energy consuming) ankle action in the landed foot.

Structural evidence for the lever action includes, among others, early electron micrographs of myosin II which is not processive (Reedy et al., 1965), crystal structures of myosin II (Rayment et al., 1993; Houdusse et al., 1999, 2000), of myosin V (Coureux et al., 2003, 2004; Holmes et al., 2003) and of myosin VI (Ménétrey et al., 2005), and electron micrographs of myosin V (Walker et al., 2000; Burgess et al., 2002). Reversal of the motor direction by an artificial design of the ankle structure (Tsiavaliaris et al., 2004) strongly supports this biasing mechanism. Tilting of a (presumably landed) leg during walking has been demonstrated for myosin V (Forkey et al., 2003; Shiroguchi et al., 2005), although these observations do not necessarily show that tilting is the cause and not a result of forward motion. That tilting is an active, force producing event is best shown in single-molecule assays with a single-foot construct. For the processive motor myosin V, tilting results in a displacement (presumably of, or close to, the hip portion) of 20-25 nm (Moore et al., 2001; Veigel et al., 2002) that can take place against a backward load of more than 2 pN (Veigel et al., 2005). Another processive myosin, myosin VI that moves in the direction opposite to most other myosins (Wells et al., 1999), shows a single-leg displacement of 12 nm (Rock et al., 2005).

For kinesin without stiff legs, lever action in its genuine sense is not possible. Rice et al. (1999), however, have shown that the flexible leg of kinesin docks onto a landed foot upon binding of ATP. This will move forward the pivot for the Brownian motion of the lifted foot, biasing its diffusion forward (Fig. 4). In effect, docking is equivalent to lever action.

2.5. Is the Lever Action Sufficient for Biasing?

It thus seems that the walking mechanism of molecular motors has basically been worked out. Strain-dependent lifting of the trail foot and a lever action in the landed foot/leg seem to warrant forward movement. Do we not miss something? The problem is this: biasing of diffusion by a lever action is unlikely to work under a high backward load.

Apparently the most serious example is myosin VI, which is processive and walks with long strides (Rock et al., 2001; Nishikawa et al., 2002), possibly the longest known of about 37 nm (Ali et al., 2004). The upper part of the legs of myosin VI is flexible over ~80 amino-acid residues with a contour length of 29 nm, and thus this portion cannot serve as a lever (Rock et al., 2005). The lower, presumably stiff light-chain binding part of the leg could form a lever, but its length (Ménétrey et al., 2005) seems to be at most ~10 nm. Yet myosin VI moves forward with step sizes of 30±12 nm against a backward load of 1.7 pN (Rock et al., 2001). The backward load, presumably applied to the hip portion, would easily pull the hip backward, without suffering resistance from the flexible leg (Fig. 5*a*). The pivot for the diffusion of the lifted leg would thus be on the rear side of the landed leg. The diffusion would be biased backward.

Kinesin with completely flexible legs faces a similar problem. The free-energy gain for docking is only a few k_BT (Rice et al., 2003). Thus, under a backward load, the

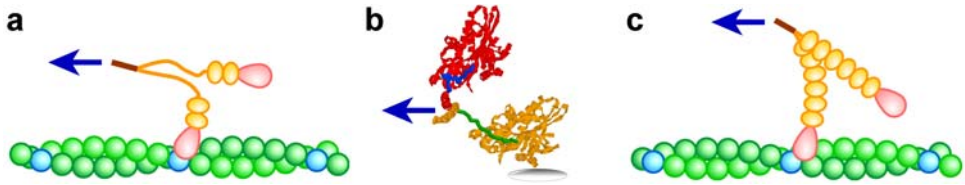

Figure 5. Moving against a backward force (arrows). (**a**) The upper part of the legs of myosin VI is flexible. Thus, a backward pull would bias the diffusion of the lifted foot toward a backward site. (**b**) The free-energy gain for docking in kinesin is only a few times k_BT. Thus, docking would readily be prohibited when the coiled-coil portion is pulled backward. (**c**) Under a backward force of 2 pN, the semi-rigid leg of myosin V is bent backward and the hip portion is pulled back by ~10 nm (Veigel et al., 2002). Forward bias for the diffusion of the lifted foot would thus be canceled.

landed leg would not be able to dock to bias the diffusion forward (Fig. 5*b*).

The legs of myosin V are stiff, but not perfectly so. Its effective stiffness is 0.2 pN/nm (Veigel et al., 2002, 2005), implying that a backward pull at 2 pN will bring back the hip by 10 nm, almost canceling the forward bias (Fig. 5*c*). Myosin V still moves forward under this load (Mehta et al., 1999; Reif et al., 2000).

There must be a mechanism, other than the lever action that moves the pivot forward, that warrants landing of the lifted foot on a forward site.

3. TOE UP-DOWN MECHANISM

3.1. Orienting the Lifted Sole Correctly

When a man lifts a foot while he walks, he immediately brings the toe down by unbending the ankle. When that foot is thrown forward, the sole will be correctly oriented for landing. Molecular motors may well adopt the same ankle action (Ali et al., 2004; Kinosita et al., 2005), because their sole must also be oriented correctly against a landing site on the track to allow stereospecific interactions between the two.

The lever action discussed above is forward bending of the ankle in a landed foot. The ankle, once bent, must be unbent in a walking cycle, in preparation for the next lever action. The unbending in this sense, for myosin, is often called ‘cocking’ or ‘priming.’ Here we propose that unbending, or bringing the toe down, plays a more positive role of warranting landing on a correct, i.e. forward, site. The ankle action in a lifted foot (Fig. 6*a*) may be equally, or possibly more, important than the ankle action in a landed foot.

Once the lifted toe is brought down, forward landing is natural because the sole is parallel to the track surface (Fig. 6*b*), whereas backward landing would introduce a considerable strain in the leg (Fig. 6*c*) and/or in the ankle. This is so even in the presence of a backward load. The load may pull back the hip portion to the rear side of the landed foot, but backward landing still results in a similar amount of strain (Fig. 6*c*), whereas smooth forward landing ensues when the hip happens to move forward by Brownian motion. Of course forward motion of the hip should be infrequent under a high backward load, but this is consistent with the known load-dependent decrease in the stepping rate of molecular motors (Mehta et al., 1999; Visscher et al., 1999; Altman et al., 2004). In the case of myosin where landing sites are available close to a landed foot, the

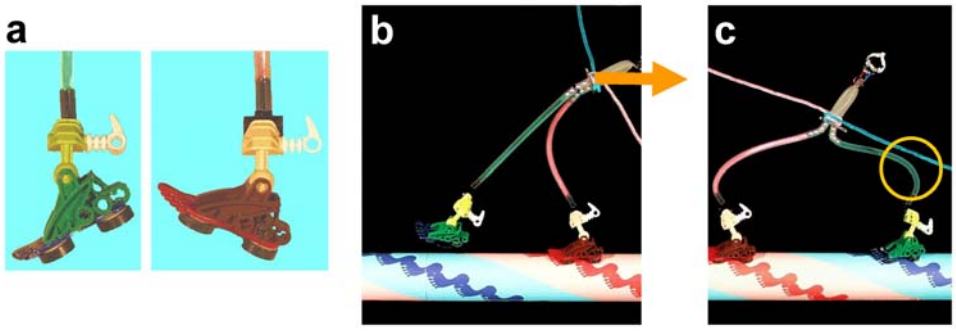

Figure 6. Ankle action in a walking molecular motor. (**a**) Ankle action, or toe up-down, changes the orientation of the sole relative to the leg. (**b**) With the lifted toe down, the sole becomes parallel to the track surface when the foot is thrown forward. Even when the hip is pulled backward (arrow), forward landing is assured, with a little bit of help by Brownian motion. (**c**) If the foot with its toe down is forced to land on a backward site, considerable strain, in the form of leg bending in the figure (orange circle), is introduced. Note that bending of the pink leg in *b* and *c* is due to the backward load (orange arrow) and does not contribute to biasing.

nearest being on the neighboring actin monomers at ±5.5 nm (see footmarks in Fig. 6*b* and *c*; landing on a neighboring monomer, though, is not completely free of steric hindrance), landing under a high backward load might be on a nearby site. The motor then effectively stalls, as observed experimentally under a high load.

A nice feature of the toe up-down mechanism is that it will operate with flexible legs, as long as landing sites are far apart compared to the leg size (Ali et al., 2004; Kinosita et al., 2005). This seems to be the case for kinesin, for which the two flexible legs need to be fully extended to straddle two landing sites that are 8 nm apart (Fig. 4*d*). As illustrated in Fig. 7, a lifted foot with its toe down can land only on the forward site, because the orientation of the sole is restricted at the end of a fully extended leg. Here again, a backward load will pull back the pivot for the Brownian motion of the lifted foot, making forward landing infrequent. Backward landing would still be difficult, as shown in the second row in Fig. 7. Note that, under a very high backward force, the toe up-down mechanism cannot warrant forward landing, but biased diffusion based on the lever action alone not only fails in warranting forward landing but would promote backward landing.

For myosin VI with two long legs that are stiff at the lower end but flexible in the upper portion, many landing sites are available on the actin track. This results in a broad distribution of step sizes (Rock et al., 2001; Nishikawa et al., 2002). With the toe down, backward landing should still be difficult, because a 36-nm back step would require an almost full extension of the legs and a shorter back step would require a bowlegged posture (Ali et al., 2004; Kinosita et al., 2005). Short steps, a few actin monomers backward or forward, may be allowed for myosin VI, but experimental detection of such short steps will be difficult.

When pulled back with a superstall force, a motor may walk backward as shown in Fig. 7, bottom. The lead ankle would unbend (undock in the case of kinesin) and/or the lead leg would somehow be stretched to allow backward landing. Backward walking has indeed been observed for kinesin (Carter and Cross, 2005), although the high backward force often ripped the kinesin off the microtubule instead of allowing continuous backward steps. A similar observation has also been made on myosin V

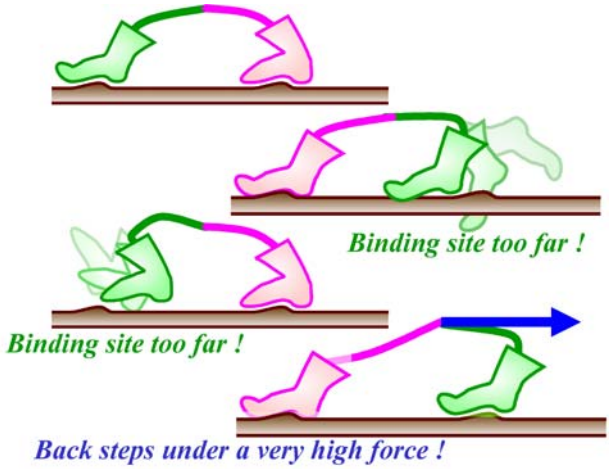

Figure 7. Toe up-down with flexible legs. If landing sites are far apart such that two-foot landing requires (almost) full extension of the two legs, with the lead toe down and trail toe up (top row), then a lifted foot (green in the second row) with its toe down cannot land on a backward site even if the other toe (pink) is also down. If, on the other hand, the toe remains up after lifting (green foot in the third row), forward landing would be prohibited. Under a very high backward load (fourth row), backward walking may take place if the lead ankle yields and/or the lead leg is somehow stretched. The backward walking will not be stable, because the lead foot tends to be detached. Recently, backward walking has indeed been observed for kinesin (Carter and Cross, 2005) and myosin V (Clemen et al., 2005).

(Clemen et al., 2005), of which the legs are semi-rigid.

3.2. Structural Evidence for Toe Up-Down

Is the toe up-down mechanism a mere conjecture? We note that crystal structures of linear motor proteins obtained so far are all without an associated track, and thus represent a structure of a lifted, not landed, foot. These structures do support the toe up-down mechanism (Fig. 8), although they are often cited as a support for a lever action in a landed foot.

In Fig. 8*a* and *b*, two crystal structures of scallop myosin (Houdusse et al., 2000) are compared. Although this myosin is not processive, structures of the processive myosin V are essentially similar to those of scallop myosin (Coureux et al., 2004). The foot in Fig. 8*a* binds ADP and vanadate which is an analog of phosphate. Myosin is supposed to bind to actin when bound ATP is split into ADP and phosphate. Thus, the structure in Fig. 8*a* represents the one prior to (and immediately after) binding to actin. As seen, the actin-binding site, or the sole, indicated by a purple bar is oriented in such a way that it faces the actin track when the leg leans backward or when the foot is thrown forward. This is a toe-down posture that warrants landing on a forward site. Fig. 8*b*, on the other hand, shows a foot without a bound nucleotide. The lever action in a landed foot in myosin is considered to be driven by phosphate (and ADP) release, and thus the structure in Fig. 8*b* presumably mimics the structure of a landed foot/leg prior to ATP-induced unbinding from actin. This structure, as seen, is a toe-up posture, which is to serve as a trail foot/leg in a two-foot landing phase.

In conventional kinesin, the docked structure (Fig. 8*d*) presumably mimics a toe-up

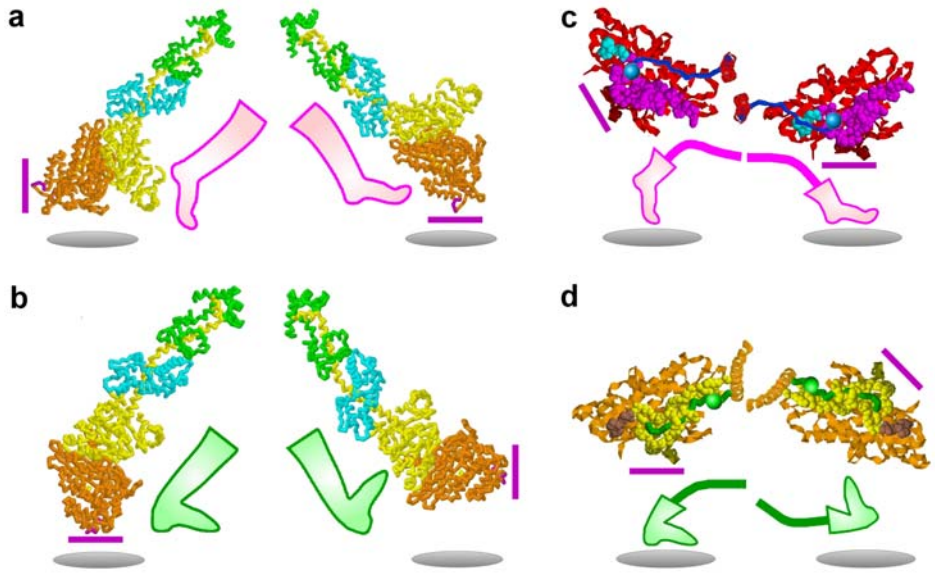

Figure 8. Structural evidence for toe up-down. (**a, b**) Myosin in a toe down (*a*) and toe up (*b*) conformation. Each panel shows two orientations of the same molecule. The leg/foot in *a* are the subfragment 1 (S1) of scallop myosin binding MgADP·VO_4, presumably representing a structure before and/or immediately after landing, and those in *b* are S1 without a nucleotide, probably mimicking a structure at the end of the lever action (Houdusse et al., 2000). Purple bars indicate the actin binding site, and gray disks represent myosin-binding sites on an actin filament. (**c, d**) Conventional kinesin with both feet in a toe down (*c*) and a toe up (*d*) conformation. The leg (neck linker) is shown in a backbone representation, and is undocked in *c* (blue) and docked in *d* (green). Atoms forming the docking site are shown in spheres (magenta in *c* and dark yellow in *d*). Cyan atoms in *c* show the position of an alternative docking site found in a related motor Eg5 (Turner et al., 2001). Large balls (blue in *c* and green in *d*) show the pivot for Brownian motion of the foot. Purple bars indicate the microtubule binding site, and gray disks represent kinesin-binding sites on a microtubule. The structures shown are one monomer in the dimeric kinesin structure (Kozielski et al., 1997), arranged in two arbitrary orientations in each panel; the α-helix in the coiled coil portion and the neck linker in *c* have been modified.

posture of a landed foot, because the docked leg (green) is extended in the direction of motor movement to position the pivot (green sphere) on the forward side of the foot. This docked, toe-up posture is so designed as to serve as a trail foot/leg, as indicated by the green cartoon in Fig. 8*d*. Upon lifting of the foot, the leg must undock to allow the lifted foot to reach the forward landing site. When undocked, the flexible leg will likely adopt all possible conformations excluding the docked conformation. Leg conformations close to the docked one will not be allowed, because of the interference with the residues forming the docking site. We consider the allowed, undocked conformations as toe-down postures (two examples shown in Fig. 8*c*): these conformations, due to the steric interference, cannot serve as a trail foot/leg in two-foot landing, and these should include at least one conformation that serves as a lead foot/leg in two-foot landing (Fig. 8*c*, right).

In an ADP-bound, and thus presumably lifted, foot of Eg5, a member of kinesin superfamily, the neck linker (leg) has been found to be docked onto a different site (cyan in Fig. 8*c*) and assume an ordered structure (Turner et al., 2001; Sablin and Fletterick, 2004). For Eg5, this conformation likely dominates the toe-down postures. A

predominant conformation(s) may also exists in the toe-down postures of other two-foot kinesins including conventional kinesin, although the residues forming the alternative docking site appear to be unique to the Eg5 subfamily (Turner et al., 2001).

4. MECHANISMS THAT WARRANT FORWARD WALKING

We have discussed four mechanisms that together propel a two-foot, processive molecular motor forward: (1) a lever action that moves forward the pivot for diffusion of a lifted foot, (2) diffusion of the lifted foot, which is biased forward by the lever action unless a high backward load is applied, (3) toe up-down that biases landing toward a forward site, and (4) strain-dependent, preferential lifting of the trail foot. Of these, 1, 2, and 4 have been widely and extensively discussed in the literature.

Here we claim that 3 is equally important, particularly in the presence of a backward load. Under a high backward load, in particular, the role of lever action may well be auxiliary: preventing the pivot from moving too far back. With motors with flexible legs, even a small backward load should be sufficient to pull the hip portion to the rear side of the landed foot, neutralizing and even reversing the bias introduced by the lever action. Correct sole orientation dictated by toe up-down, evidenced by crystal structures, seems to be of vital importance in warranting forward walking. Quantitative appraisal of the toe up-down mechanism, such as the energy difference between the up and down postures or the dependence of diffusion and landing kinetics on the up/down states, awaits further studies.

In this article, we have focused on processive motors that have two identical feet. There are also one-foot motors, such as an unconventional kinesin KIF1A that moves processively along a microtubule, though not steadily in one direction (Okada et al., 2003), or myosin IXb (Inoue et al., 2002). The motion is essentially a one-dimensional biased diffusion on a filamentous track. Biasing in one direction requires force, as stated at the beginning. Production, within a motor, of a force that moves the motor forward, as opposed to a force that simply drives an internal conformational change, requires that part of a motor is firmly attached to the track against which the force operates. Thus, when a one-foot motor is in a biasing phase where the motor moves unidirectionally forward, the motor likely undergoes the following sequence: (i) one part of the motor is bound to the track, (ii) another part binds, resulting in two-spot binding, and (iii) the first part detaches, resulting in a net movement forward. This sequence is essentially the same as that in two-foot motors, except that the two binding events are not equivalent and that the motor may proceed to a diffusing phase after iii. Indeed, KIF1A has been shown to use two microtubule-binding loops alternately to bias its diffusion forward (Nitta et al., 2004). Conventional two-foot kinesin may also use the same tactics in a landed foot to move itself forward. That would be a lever action in the sense used in this article, in that it will move the pivot forward. Docking may not be the only way of moving kinesin' pivot forward.

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