Not So Lame After All: Kinesin Still Walks with a Hobbled Head

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It's no secret to electrophysiologists that single-molecule methods have driven some of the most impressive advances in our understanding of how biomolecules function. In fact, the power of single-molecule techniques had become abundantly clear by the mid 1980s, when a review of patch-clamp results noted "It is now routine to observe the behavior of one protein molecule with a time resolution approaching 10 μs. Amazing!" (Auerbach and Sachs, 1984). Further technological developments have made single-molecule methods available to a growing range of biophysical subfields, including the study of motor proteins, or mechanoenzymes (Block et al., 2007). As the techniques have become more robust and reliable, many of the key biochemical tools that have long been exploited in ensemble-averaged experiments, such as use of small-molecule inhibitors, are finding their way into single-molecule motility assays. A new report by Subramaniam and Gelles (on p. 445 of this issue) signals this growing trend by describing novel behaviors of single kinesin proteins in the presence of adenylyl-imidodiphosphate (AMP-PNP), a nonhydrolyzable analogue of ATP known to inhibit kinesin's catalytic activity. Surprisingly, the authors found that kinesin motors could still move when one of its twin heads was hobbled by the analogue.

Since the discovery in 1985 of kinesin, an intracellular cargo transporter (Brady, 1985; Vale et al., 1985), our knowledge of its structure and mechanism has progressed at a stunning pace. Conventional kinesin (kinesin-1) consists of two catalytic domains (heads) that dimerize together via a common, coiled-coil stalk (Amos, 1987). Kinesin moves processively, translocating along microtubule tracks at velocities in the range of 0.5–1.0 μm/s over distances of 1 μm or so before dissociating (Block et al., 1990). The two head domains move alternately, in a "hand-over-hand" fashion as the molecule advances in discrete steps of 8 nm (the tubulin dimer repeat distance along a microtubule protofilament), hydrolyzing one molecule of ATP in concert with each of its steps (Svoboda et al., 1993; Hua et al., 1997; Schnitzer and Block, 1997; Asbury et al., 2003; Kaseda et al., 2003; Yildiz et al., 2004). A carefully orchestrated coordination between the mechanical and chemical cycles of the two heads is somehow responsible for its remarkable processivity.

Early mechanistic studies explored the specific structural elements responsible for kinesin processivity. Mutant kinesin constructs engineered to consist of a single head, missing the stalk or a partner head, were catalytically active but generally lacked processivity (Berliner et al., 1995). Two heads are therefore required for processive motion. Several subsequent studies showed that the heads carry out a hand-over-hand walk, alternating taking leading and trailing positions, as the motor moves toward the plus-end of the microtubule (Asbury et al., 2003; Kaseda et al., 2003; Yildiz et al., 2004). To coordinate such a walk, the trailing head must always release from the microtubule before—and not after, or concomitant with—the leading head. This requirement implies that the catalytic cycles of the heads are mutually "gated'" in some fashion. Without gating, nothing would prevent the premature termination of a processive run, caused, for example, whenever both heads simultaneously release from the microtubule. Nothing would prevent frequent backsteps, either, caused by release of the leading, rather than trailing, head from the microtubule. Moving without the coordination imparted by gating would be a bit like trying to walk on an icy pavement—there would be no guarantee that your foot would move where or when you wanted, causing you to stagger or fall down. If a wind were blowing hard enough, you might even wind up going backward. So, too, an ungated kinesin molecule might move only backward in the presence of rearward loads. The prevailing assumption has been that both heads must remain catalytically active for gated stepping to take place. However, Subramanian and Gelles (2007) now show that this need not be the case. They report that when one of the two kinesin heads is poisoned by the inhibitor AMP-PNP, the entire molecule is still capable of weakly processive motion, suggesting there may be an alternative mechanochemical cycle that supports coordinated stepping.

Subramanian and Gelles used video microscopy to score the motions of small beads attached to single molecules of dimeric kinesin. In general, the Brownian motions of such beads tend to obscure the nm-scale displacements produced by the motor itself, and therefore make it difficult to record high-precision data. Using an optical trap to record kinesin-driven bead motions can suppress some of

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Abbreviation used in this paper: AMP-PNP, adenylyl-imidodiphosphate.

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this noise, but the trap also applies loads to the kinesin molecule and therefore modifies its kinetic properties. The measurement of kinesin motility in the absence of load therefore poses quite a challenge, and Subramanian and Gelles have risen to it, by returning to an earlier video-tracking technique which, when carefully implemented, allows their particle-tracking algorithm to reliably detect kinesin motions as small as 3–4 nm within a single video frame time (33 ms) (Gelles et al., 1988).

At the time of its discovery, kinesin was distinguished and readily purified from other cellular components thanks to its unusual property of binding tightly to microtubules in the presence of the nucleotide analogue AMP-PNP (Lasek and Brady, 1985). Previous single-molecule measurements, conducted by both the Gelles laboratory and our own, showed that the binding of AMP-PNP to kinesin induces lengthy pauses, typically lasting seconds, in records of kinesin stepping (Vugmeyster et al., 1998; Guydosh and Block, 2006). This scenario is akin to the situation of a hiker getting a boot stuck under a rock on the trail, halting forward progress. The hiker is free to reposition the opposite leg forward and backward in an attempt to free the stuck boot, but is unable to make net progress until the boot comes free. We reported evidence for this sort of back-and-forth motion in an earlier optical-trapping study of kinesin pauses induced by admixtures of ATP and AMP-PNP or another inhibitor, BeF_x (Guydosh and Block, 2006). A key finding to emerge from our study was that the inhibitor could only be released when it was bound to the front kinesin head, and not the rear. This conclusion supports the growing view that the front head binds or hydrolyzes ATP more slowly than the rear head, and therefore supplies evidence for an asymmetry in head domain properties that's implicit in the concept of gating (Rosenfeld et al., 2003; Klumpp et al., 2004).

In the experiments reported by Subramanian and Gelles, single-molecule records were again obtained in the presence of admixtures of ATP and AMP-PNP, and the pauses were scored. Unfortunately, video records acquired under unloaded conditions lack sufficient resolution to resolve short-lived backsteps directly, similar to those we had observed in the presence of hindering loads. Subramanian and Gelles circumvented this limitation following the time-honored practice of single-channel electrophysiologists to examine the lifetime distribution for the pauses. It was thus possible to provide indirect evidence for multiple (unresolved) states during pauses, because the lifetime distribution for pauses was fit by a triple exponential decay, implying the existence of at least three distinct paused states. What's intriguing is that kinesin could populate one of those three states only in the presence of high concentrations of AMP-PNP. The favored interpretation of this result is that a second AMP-PNP molecule can bind to the other head of a paused kinesin molecule (initially halted by the first molecule of AMP-PNP), but only when the concentration of AMP-PNP is sufficiently high. The second binding site would therefore have weaker affinity for AMP-PNP. Assuming that this interpretation is correct, it supports the notion that one of the two kinesin heads, most likely the front, has a greatly reduced affinity for ATP analogues. In principle, confirmation of such a binding asymmetry might be obtained in future single-molecule or ensemble quenched-flow experiments using fluorescence or other spectroscopic techniques to quantify the binding stoichiometry.

Even more surprising, the authors found that the distances moved by kinesin molecules between analogue-induced pauses arose from one of two distinct populations, which they called "short" and "long" runs. The average distance traversed by a short run was independent of the AMP-PNP concentration, whereas the average distance for a long run was concentration dependent. This suggests that the long runs correspond to normal processive motion, where both heads hydrolyze ATP during hand-over-hand stepping. Consistent with this interpretation, the velocity during long runs was identical to that of kinesin stepping in the absence of AMP-PNP. Such runs pause when one of the heads binds an AMP-PNP molecule. The short runs, in contrast, do not require AMP-PNP binding to enter the paused state, implying that the AMP-PNP analogue may still remain bound to the kinesin molecule during a short run—that is, while the molecule does (limited) processive stepping! Despite the apparent impairment of one of the two heads, the velocity of the motor during a short run was slowed by, at most, a factor of 4 below that typical of normal stepping. To be sure, though, the run length declined drastically, from 800 nm during long runs to just 12 nm, on average, during short runs (for [ATP] = 0.5 mM and [AMP-PNP] = 0.05 mM). Although a few of the short kinesin runs managed to cover a sizeable distance, occasionally reaching 96 nm ($\sim$12 steps), most kinesin molecules only eked out a step or two before pausing again.

The proposal that a kinesin molecule might continue to step processively despite having one of its two heads catalytically inhibited seems likely to generate controversy. It challenges the prevailing wisdom, because hand-over-hand stepping requires that the heads alternate between weak and strong affinity for the microtubule as these exchange leading and trailing positions. Kinesin heads attach strongly to microtubules whenever they have ATP or ADP-P_i bound, or possess an empty nucleotide pocket (rigor). However, once ATP is hydrolyzed and P_i gets released, a head having only ADP bound has only a weak affinity for the microtubule, and is therefore free to move to the next microtubule binding site during the stepping transition. The accepted view was therefore that AMP-PNP, acting as an ATP analogue, could only induce heads to bind tightly to the microtubule. So what,

then, corresponds to the weakly bound intermediate state that permits processive stepping?

There may be some ways out of this conundrum. One is that the head carrying AMP-PNP never actually comes free of the microtubule, so that kinesin molecules diffuse, in effect, along the microtubule surface. An analogous diffusion mechanism has been proposed to explain the motility in vitro of single-headed recombinant constructs of the kinesin-3 motor, KIF1A (Okada et al., 2003), which is weakly processive. However, that possibility was ruled out by Subramanian and Gelles, who noted that all the forward motion they observed seemed to consist of high-duty-ratio stepping, rather than diffusive drift, as evidenced by the unidirectional character of bead motions; this is distinct from the kinesin-3 observations.

The heretical notion that kinesin molecules might somehow be hydrolyzing AMP-PNP on the timescale of short runs seems to be excluded as well, by the observation of multiple steps in the data. After releasing the products of any putative AMP-PNP hydrolysis, the motor would have to preferentially bind additional AMP-PNP molecules to continue stepping in the presence of ATP, which is thought to bind with much higher affinity. This mechanism also requires the lengths of short runs to depend upon the concentration of AMP-PNP, contrary to observation. Furthermore, kinesin does not move when ATP is removed from the assay buffer.

Another way out might be for the ATP-bound head to expend some additional energy to propel its poisoned partner. In this scenario, the head bound by AMP-PNP would display a moderate affinity for microtubules. That affinity would have to be intermediate between that of the tight- and weak-binding states adopted during the normal ATPase cycle. When AMP-PNP binds to the rear head, the affinity must be sufficiently weak to favor unbinding of the rear head before the front head. When AMP-PNP binds to the front head, however, the affinity must be sufficiently strong that the rear head releases first. This hierarchy of affinities would ensure that the rear head always lets go of the microtubule before the front, meeting a basic requirement for processive stepping. ATP hydrolysis, catalyzed by the uninhibited head, would power all forward motion and be gated by the inhibited partner head. This mechanism is consistent with the reaction pathway in Fig. 5 D of the paper by Subramanian and Gelles. Interestingly, motion under these circumstances would occur with a higher "fuel economy" (one ATP per two steps) than normal processive stepping (one ATP per step). This improved efficiency must come with a price. Much as a fuel-efficient subcompact cannot tow as big a trailer as a gas-guzzling pickup, a kinesin molecule stepping while bound to AMP-PNP ought to stall at a lower load than one doing normal stepping. The load is linearly related to the energy (because work = force $\times$ distance), so the stall force should be halved. Kinesin molecules typically stall at $\sim$6 pN, implying that short runs ought to stall at $\sim$3 pN. The stall force for short runs is presently unknown, but a valuable experiment suggested by the work from the Gelles lab would be to measure this quantity. A propos of this, in our prior study, we examined stepping in the presence of AMP-PNP under higher loads, ranging from 3 to 6 pN, and did not observe evidence of short runs, which is consistent with this explanation.

What remains missing is some overarching mechanism that can explain the curious switching observed between stepping and paused states. Load seems likely to bias this alternation of states, because short runs are not found at higher loads, but the dependence of short, AMP-PNP–dependent runs on other variables—such as temperature, the presence of other nucleotides (like ADP), or buffer conditions—remains to be explored, and may help to shed some light on the mechanism.

A feature anticipated for AMP-PNP–dependent short runs is that stepping records might show that kinesin molecules "limp," because the even- and odd-numbered steps are expected, a priori, to take different times. This asymmetry arises because the two heads undergo distinct chemical cycles in a mechanism that involves one good head propelling its hobbled partner; the uninhibited head repeatedly hydrolyzes ATP, cycling through states with ADP-P_i and ADP bound, whereas the inhibited head remains bound to a single nonhydrolyzable analogue, AMP-PNP. While limping was not apparent, the data lack sufficient resolution to rule it out altogether. The possibility of limping deserves a closer look using improved techniques, and any load dependence might be revealing.

So, where do we go from here? For starters, it would be helpful to know whether AMP-PNP truly remains bound during stepping, and how many molecules are bound. One way to answer that question definitively would be to observe single-molecule kinesin motion in the presence of fluorescently tagged AMP-PNP during both short and long runs. Other questions lead to even more experimental opportunities, such as: What structural states are adopted by kinesin during short runs and pauses? Does stepping in the presence of AMP-PNP still correspond to a hand-over-hand mechanism? Does the inhibitor preferentially bind to the front or rear head while kinesin is paused, and does its partner make back-and-forth steps? Does the inhibitor-bound rear head lift free of the microtubule while the front head waits for ATP to bind? Do any other nucleotides or nucleotide analogues produce similar effects?

The findings of Subramanian and Gelles may also be relevant to recent discoveries about members of the kinesin superfamily that are intrinsically heterodimeric, such as Kar3/Vik1 and Kar3/Cik1. These motors are formed through the dimerization of two different polypeptide chains. The head formed by one polypeptide has been

found to be catalytically active, whereas the other is catalytically incompetent (Chu et al., 2005; Sproul et al., 2005; Allingham et al., 2007). For the case of Kar3/Vik1, the head that was incapable of ATP hydrolysis was nevertheless reported to bind microtubules and gate the activity of its catalytically competent partner head. In light of the Gelles lab results, a homodimeric kinesin with one head inhibited by AMP-PNP might serve as a useful model system for heterodimers that are otherwise difficult to study, owing to their lack of processivity and tendency to depolymerize microtubules.

The idea that AMP-PNP binding might stabilize more than one alternative state is significant, but it raises more questions than it answers. What we can take away is that an admixture of AMP-PNP and ATP may be capable of eliciting two very different types of behavior: one where kinesin dimers stick to microtubules and fail to advance, and another where they can take a few steps processively, despite retaining the inhibitor on one head. What makes kinesin favor one type of behavior over the other, and what mechanisms underlie the associated kinetics, remains a mystery. However, this wouldn't be the first time that kinesin has left us scratching our heads and planning another round of tantalizing, single-molecule experiments.

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