Kinesin Motor Mechanics: Binding, Stepping, Tracking, Gating, and Limping

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This critical review was motivated by the 10th Biophysical Discussions meeting, ''Molecular Motors: Point Counterpoint'', held in Asilomar, CA, during October 19–22, 2006. Biophysical Discussions are meetings that focus on cutting-edge or emerging topics in biophysics that can benefit from intense discussions. Streaming videos of the speaker presentations at this conference, including a synopsis of this review, are available through the Biophysical Society's web site at http://www.biophysics.org/discussions. In keeping with the spirit of a discussions meeting, I present here a personal perspective on the current state of kinesin motor mechanics. Nearly a generation has passed since the discovery of the motor named kinesin (1), and the subsequent development of the very first single-molecule gliding-filament and bead assays for motility (2,3), which helped to establish the modern field of single-molecule biophysics. Discrete steps of single molecules were first measured for kinesin (4), followed shortly thereafter by reports of similar steps for myosin (5,6). Since then, literally thousands of single-molecule experiments have been performed on a whole variety of molecular motors, all with the aim of discovering how these remarkable protein machines function. Considerable and impressive progress has been achieved, but key questions still abound, and this remains a very lively field of endeavor. I discuss below my current thinking on several questions concerned with kinesin mechanics, listed in no particular order of precedence. I wade into controversy holding no illusions that everyone will share my views on the answers to these questions, but I do hope to provoke a more thoughtful examination, and set the record straight on at least a few points. By choice, and in keeping with the topic of the meeting session where this was presented (''Motor Walking Mechanisms''), the questions that I've posed relate directly to the nanoscale mechanics of kinesin motion. However, these same questions are intimately and inevitably linked to other aspects of kinesin structure, biochemistry, and cellular function.

DOES KINESIN TAKE SUBSTEPS? IF SO, OVER WHAT TIME AND DISTANCE SCALES?

In our original article describing single kinesin stepping, the steps were found to subtend a distance of 8 nm, and they took place instantaneously on the time scale of the experiment. Here, the data acquisition rate was 1 kHz (after anti-alias filtering at the Nyquist frequency, 0.5 kHz), and records were software-filtered to 200 Hz, for a characteristic time of 5 ms (4). Quite a number of models to explain kinesin motion have since been entertained, which predict that the 8-nm step should be composed of substeps of one form or another. Substeps are by no means unreasonable to contemplate, for a variety of plausible reasons (see below).

Two studies have claimed to identify substeps within the kinesin cycle. I don't believe that either article presented a sufficiently compelling case that substeps exist. In both instances, there appear to have been similar flaws in methodology. The first article, a collaborative effort by Vale and Spudich (7) reported the existence of a comparatively long-lived intermediate state during the forward step, lasting on the order of 10–20 ms, which separated the 8-nm step into two distinct components of 5- and 3-nm (with the 5-nm component being the most clearly resolved). However, the starting and ending points of the steps in data records were a), scored entirely ''by eye'' from b), traces filtered with a 15-ms median filter. Under these circumstances, no statistically meaningful plateaus can exist whose characteristic times are comparable to that of the smoothing filter (15 ms). Although the data were sampled at 2 kHz, this did not mean that they were trustworthy at a data interval of 0.5 ms, because the bandwidth of the analog position signal was limited to 110 Hz, corresponding to a characteristic time of 9 ms. In retrospect, it seems likely that the milliseconds-long plateaus seen in the noisy records were the consequence of a data selection artifact. Since 1996, the time resolution for the routine recording of kinesin stepping has steadily improved, particularly for smaller beads subjected to higher loads, where it now routinely achieves $\sim$1 ms or better (see, for example, (Guydosh and Block (8)). No group has ever duplicated these findings.

The second article, the result of a collaborative effort by the Yanagida and Higuchi labs (9), achieved substantially higher temporal resolution, and reported substeps lasting on the order of 50 μs, some 200-fold faster than those reported by Coppin et al. (7). Here again, though, the same two issues resurface, associated with a), data sampling by selection and b), a failure to assess the effects of instrument bandwidth. Because of the presence of noise, individual records of steps showed no clear evidence of substeps. However, a subset of records displayed small fluctuations (seen as plateaus) during their rising phase for a step: these records were separated from those that rose more smoothly (again, ''by eye'') and placed in two further

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batches, with plateaus lasting either 50–100 μs or >100 μs, then separately averaged together. Such a selection procedure, followed by averaging, seems guaranteed to reinforce any random fluctuations (noise) that may have contributed to the plateaus, along with genuine signals (if any). The traces with apparent 50–100 μs plateaus seemed to divide the 8-nm step into two equal components of 4 nm. However, although data were acquired at 100 kHz using dark-field laser illumination onto a quadrant photodetector, signals had been passed through a 20 kHz analog low-pass filter before digitizing, so the characteristic response time of the measurements was 50 μs. This time is remarkably similar to their measurement of the average time constant for the abrupt rising phase of a step (Fig. 3 of Nishiyama et al. (9)), which came to 48 μs. It is not meaningful to extract timing information in the ''10 μs range'' when instrument response times are restricted to comparable intervals.

More recently, Cross's group has reinvestigated the question of kinesin substeps, and reported finding no evidence for these down to their experimental cutoff time, estimated to at ~30 μs (10). In their case, the measurement system, based on bright-field imaging onto a quadrant photodetector, had a combined bandwidth of 46 kHz (~21 μs), but in most cases data were sampled at 80 kHz and averaged down to 20 kHz (~50 μs) for analysis. The effective bandwidth is therefore quite comparable to that of the instrument employed by the Yanagida and Higuchi group. However, steps were scored here by an automated algorithm, and not binned by eye into categories for subsequent averaging. My own group has also sought evidence of kinesin substeps. In unpublished work, we found no evidence for these with an instrument that uses back focal plane detection of scattered laser light onto a position-sensitive detector. Our photodetection subsystem has an analog bandwidth of ~200 kHz, but the computer data acquisition was limited to ~35 kHz, corresponding to a characteristic system response time of ~30 μs. We concur with Carter and Cross (10) that no substeps can be found down to this response time, and steps are still instantaneous on the timescale of our measurements.

None of this is to say, however, that kinesin substeps don't exist! The Yanagida group has argued that the size of the ''characteristic distance'', δ, for kinesin movement (11), a parameter that can be derived from force velocity curves, implies the existence of substeps, given that its value is ~3 nm, which is only a fraction the full 8-nm step (12). However, I do not accept that argument as being decisive. As we have previously noted in Wang et al. (13), the physical interpretation of the characteristic distance, δ, is highly model-dependent, and several very different classes of biochemical pathways can lead to force-velocity relationships with a similar Boltzmann-type shape. In some of these pathways, the characteristic distance corresponds directly to a measurement of the step size (14), but in others, it corresponds instead to the distance to a transition state, which is always less than the step size. It therefore seems possible that a value of δ ~3 nm could be reconciled with either full-stepping or substepping pathways; additional evidence is required to decide the issue.

Could substeps be accommodated? Yes, provided these are exceedingly short-lived. An unloaded kinesin head can diffuse over 8 nm in a time of ~10 μs (based on approximating the head as a 10-nm diameter sphere diffusing in water through 8 nm, according to $\langle x^2 \rangle \approx 2Dt$). However, this first-passage time rises exponentially fast when the head is forced to move against a load of any size (15). If the actual kinesin step consists, for example, of a), an initial conformational change followed by b), a diffusional component that carries the head the remainder of the way to its next microtubule binding site, then it seems possible that evidence for substeps may be very difficult to discover, in practice. That difficulty would be exacerbated if the distance subtended by the conformational component constituted a comparatively small fraction of the overall step (say, ~2 nm, measured at the common stalk joining the heads) and the diffusional distance is larger.

WHAT'S THE KINESIN WALKING PATTERN (''WADDLE MODEL''), AND WHAT DO WE LEARN ABOUT ITS MECHANICS FROM THIS?

At least four single-molecule experiments bear directly on this question (16,17,18,19). The Gelles lab (16) found that the short kinesin stalk of a recombinant *Drosophila* construct (K448 with a C-terminal biotinylation site) was torsionally rigid, a finding that contrasted sharply with earlier measurements of the stalk from full-length bovine kinesin, which was found to be surprisingly flexible overall, permitting k_BT of energy to twist the stalk by more than one full rotation (20). The rigidity of the short recombinant stalk allowed them to track the rotational Brownian motion of microtubules moved by single kinesin molecules. That movement was found to be tightly bounded, and did not produce large angular motions of 180° or more during stepping motion. In their article, Gelles and co-workers introduced important terminology for three different types of kinesin walk: symmetric hand-over-hand (where the two heads exchange leading and trailing positions on the microtubule, but the three-dimensional structure of the kinesin molecule is preserved at all equivalent points in the step cycle), asymmetric hand-over-hand (where the kinesin heads exchange positions on the microtubule, but the initial and final states of the molecule are not symmetry-related, implying that alternate steps must differ in essential ways), and inchworm (where one head always leads and the other always trails during the cycle of advancement; all inchworm models are necessarily symmetric). The failure to observe large angular changes in the stalk ruled out the symmetric hand-over-hand (HoH) model, which would have produced 180° stalk rotations. The body of evidence was therefore interpreted as favoring the inchworm model. However, as Hua et al. were careful to point out, the asymmetric HoH model could not be ruled out altogether by their data, although it would place severe constraints on the ways in which the

molecule might move between stepping states. They wrote: ''Thus, although our experimental results do not rigorously exclude an asymmetric hand-over-hand mechanism, we regard as improbable the existence of two structures that simultaneously satisfy all of the requirements outlined above.''

The subsequent discovery of ''limping'' in kinesin, where the average kinetics of every other step switch between a faster and a slower stepping phase, proved that kinesin dimers advance through an asymmetric HoH motion, and that this motion is inconsistent with either the inchworm or symmetric HoH patterns. This is because kinesin dimers were found to alternate between two distinct (identifiable) states with each step, precisely as required by the asymmetric HoH model, which alone breaks symmetry: no such alternation can exist in either the (symmetric) inchworm or symmetric HoH models. Limping kinesins were generated in two rather distinct ways, using recombinant constructs of *Drosophila* kinesin. Work by Kaseda et al. (18) produced heterodimers with one ''wild-type'' head and the other head slowed by a mutation to the nucleotide binding pocket (R14A), which reduces the microtubule-stimulated ATPase rate by nearly 20-fold. Independent work by Asbury et al. (17) found that appropriate homodimer constructs of kinesin would also limp, provided that their stalk regions were sufficiently short. In fact, the degree of limping was found to be anti-correlated with the length of the stalk.

Reports of kinesin limping were very soon followed by some compelling experiments from Paul Selvin's group that followed the motion of an individual dimeric kinesin head labeled by a single fluorophore, using video centroid tracking accurate to nearly 1 nm (19). Kinesin heads (with labels on the heavy chain placed sufficiently close to the head domain) appeared to advance in a series of ~16 nm steps, a result consistent with HoH motion but inconsistent with inchworm motion, which would have produced ~8 nm steps instead. Importantly, however, and in contrast to the two earlier limping experiments, the centroid-tracking experiments do not distinguish between symmetric and asymmetric HoH motion, a fact that seems to have eluded more than one review writer. Modeling of biochemical kinetic results by Schief et al. (21) also supported HoH motions, as opposed to inchworming. Because the results of the Selvin lab support either symmetric or asymmetric HoH stepping models, whereas the results of the Gelles lab support either inchworm or asymmetric HoH models, the only stepping pattern consistent with both sets of results is asymmetric HoH motion. This, of course, is fully consistent with the two limping reports, which unambiguously indicated asymmetric HoH motion. All in all, the body of evidence in favor of the asymmetric HoH model is very compelling.

We still don't know what causes limping in homodimer constructs, but our experimental results suggest that it is unlikely to be simply an artifact of the linking geometry to the bead itself. Kinesin homodimers with short stalks limp whether bound to beads by streptavidin- or by antibody-based linkages. The degree of limping correlates with the length of the stalk and the value of the external load, and is most pronounced when the load is highest. This result is not consistent with some form of nonspecific interaction between one of the heads and the bead, an interaction that would be destabilized (and therefore diminished) at higher loads; this explanation therefore gives the wrong sign for the load-dependence. Moreover, if one head were to interact transiently with the bead for a significant portion of the cycle (as required for this explanation to hold), then the position of the bead would tend to report the position of a single head, rather than the centroid of the molecule (the stalk position), leading to alternating step sizes as well as step timing, contrary to observation. Dimers that are cross-linked by disulfide linkages between cysteines introduced into the proximal dimerization domain at the base of the stalk continue to limp, suggesting that helix misregistration of the coiled-coil region cannot be responsible for the phenomenon (Block lab, unpublished data). However, there are several other candidate explanations that are currently under test, and some of these involve torsional effects of the heads with respect to the stalk.

Given the body of evidence in support of an asymmetric HoH stepping pattern, an obvious question arises as to how symmetry is actually broken for kinesin, which surely involves the microtubule itself. A corollary of the asymmetric HoH walk is that there must be two intrinsically different kinds of steps taken by kinesin molecules (call these a ''left'' step and a ''right'' step), and that these steps differ in both their trajectories (i.e., in the underlying molecular geometry) and also in their biochemical kinetics. Notwithstanding, the left and right kinesin steps are generated by head domains that are nominally identical in amino acid sequence (at least for homodimers), and the same head can generate either a left or a right step depending on its microenvironment. The consequences of this are far-reaching and profound, I believe.

HOW DO THE TWO KINESIN HEADS MANAGE TO STAY OUT OF PHASE WITH ONE ANOTHER DURING THE STEPPING CYCLE (I.E., HOW ARE THEY ''GATED'')?

The temporal sequencing involved in stepping requires some form of communication between the heads to synchronize their biochemical cycles in precisely such a way as to maintain them out of phase, or else processivity would rapidly be lost. Furthermore, the evidence that kinesin's 8-nm step is tightly coupled to the hydrolysis of a single ATP molecule (22,23,24) also implies some form of coordination between the cycles of the two heads. In fact, kinetic data on single-headed motors support the notion that processivity derives from head coordination (25,26). The only realistic basis for such a gating mechanism would seem to be the mechanical strain that develops between heads during stepping itself. In principle, there are two plausible candidates for communicating this strain: through the regions joining the two kinesin heads, i.e.,

the neck linker regions and the common stalk, or from the heads through the microtubule protofilament. Of course, these are not mutually exclusive. Furthermore, whenever discussing the effects of strain on movement, one must remain mindful of the inherent reciprocity between the mechanics and the biochemistry: the load can affect the binding and hydrolysis, but binding and hydrolysis equally well affect the forces generated. These are intimately linked.

Broadly speaking, two general classes of gating mechanism have been entertained. In one (the so-called ''gated rear head'' mechanism), the mechanical release of the trailing head from the microtubule leading head is accelerated by internal strain (27). Experimental support for this picture comes from the work of Crevel et al. (28) and Schief et al. (21), who reported that strain accelerates the detachment rate of the rear head. In the other model (the so-called ''gated front head'' mechanism), ATP binding to the leading head is suppressed through internal strain (29,30). Note that these are not mutually exclusive, either, so mixed models are feasible. Work on head unbinding forces by the Ishiwata group has also helped to establish the notion that kinesin's affinity for nucleotide is dependent on the directionality of an external load, and the apparent K_D of a kinesin head for ADP is weakened up to sevenfold for rearward versus forward load (31).

Additional evidence supporting a gated front head mechanism comes from recent work by Guydosh and Block (8) on the effects of nucleotide analogs (AMP-PNP and ADP·BeF_x) on single-molecule motion driven by ATP. The addition of low concentrations of these nonhydrolyzable analogs causes stepping kinesin molecules to enter into long pauses, until the analogs can be released and ultimately exchanged for ATP. After a pause induced by an analog, it was discovered that processive stepping could only resume once the kinesin molecule took an obligatory, terminal backstep, exchanging the positions of its leading and trailing heads, which allows release of the bound analog from the (new) front head. Preferential release of the analog from the front head, as opposed to the rear head, implies that the kinetics of the two heads are differentially affected when both are bound to the microtubule. Kinesin, then, would seem to be the proverbial ''back seat driver'', where the passenger head in the rear directs the driver head in the front!

WHERE IN THE KINESIN BIOCHEMICAL PATHWAY IS FORWARD MOTION PRODUCED?

According to Hancock and Howard (27), release of stored strain upon unbinding of the trailing head permits the leading head to power an 8-nm advance of the entire molecule. According to Rice et al. (32), ATP binding induces the docking of the neck linker on the leading head to produce motion of the partner head. My own group has found that the effective binding rate for ATP is load-dependent, which indicates that ATP binding, or a transition closely coupled to it, generates the forward step (33). When taken together with other biochemical results, modeling of our data suggests that ATP binding is highly reversible and followed by some kind of conformational (and less reversible) change, leading to a mechanical step broadly consistent with the model of Rice et al. (32). The recent finding by Guydosh and Block (8) that the duration of the terminal backstep before the resumption of forward movement (from a pause induced by a nucleotide analog) depends on ATP concentration strengthens the case for a mechanical step triggered by ATP binding, and further argues against the alternative picture that the release of strain permits a step.

IS THE BACKSTEPPING CYCLE A REVERSAL OF THE FORWARD CYCLE, AND DOES KINESIN GENERATE ATP UNDER SUPER-STALL LOADS THAT FORCE IT TO MOVE BACKWARD?

Occasional backsteps have been reported since the very first studies of kinesin stepping under load (34), and their relative frequency—but not necessarily their duration—clearly depends on the applied load, because (trivially!) the forward and backward single-molecule stepping frequencies must exactly balance at stall, when velocity drops to zero. Most often, backsteps are solitary, flanked by forward steps on either side in records of processive motion. The dependence of backstepping phenomena on ATP levels, and their interpretation, must be considered controversial for the present. The frequency of backstepping did not appear to be very dependent on [ATP] in the work of Nishiyama et al. (12), although the durations of backsteps were, and these findings were interpreted in terms of a biased Brownian ratchet model. The authors went so far as to suggest that the effective temperature of the motor protein would reach 834 K (536°C), which seems preposterously high, especially in view of the fact that proteins cannot remain out of thermal equilibrium with their surrounding milieu for so much as a microsecond at a time, which is less than the time required to complete an 8-nm step by diffusion. Backstep rates were also reportedly independent of load, a result later confirmed by Carter and Cross (10), who extended this result to the regime of larger, super-stall forces, which were discovered to induce processive backstepping. Their recent experiments also found that the dwell times for both forward and backward steps decreased with increasing [ATP], suggesting that ATP binding is a requirement for both forward and backward stepping (but not necessarily its hydrolysis).

Is processive backstepping simply movement in reverse, and could ATP possibly be synthesized during load-induced, processive backstepping? Carter and Cross tend to think not, and they suggested that there ''is at present no evidence that backward stepping is coupled to ATP turnover.'' Hackney (35) has pointed out that the product of the kinesin stall force (~7 pN) and step size (8 nm) is less than the energy that he estimated to be released during ATP hydrolysis at physiological ATP, P_i, and ADP levels (87 pN nm), so a stall is not an equilibrium state. He suggests that backsteps are therefore unlikely to represent a simple reversal of the kinesin pathway. However, it

may be useful to examine more carefully the rearward motion of kinesin molecules at forces only slightly in excess of stall to see if the experimentally observed behavior is truly incompatible with the energetics of ATP synthesis. One cautionary note: Fisher has pointed out that, unlike the relative frequencies of forward and rearward stepping, which may in principle be modulated by ATP concentration, the average dwell times for forward and rearward steps are generally coupled, and these must always rise or fall together with changing ATP levels (36). The data in Fig. 5 of Nishiyama et al. (12) seem broadly consistent with this requirement (at first glance).

CONVERSELY, WHEN KINESIN IS SPED UP BY AN ASSISTING FORCE, IS IT GOING THROUGH ITS NORMAL BIOCHEMICAL CYCLE OR BY SOME OTHER PATHWAY?

The original report that kinesin could be sped up by as much as threefold beyond its normal unloaded velocity in response to external forward loads (37) is no longer considered credible, and in retrospect seems likely to have been an artifact of experimental geometry (which may have allowed kinesin to release and ''skip'' forward), and the manner in which loads were applied, which did not include force-clamped conditions. However, kinesin does speed up moderately under forward loads, and this is particularly true at low ATP levels, below the apparent K_M for movement (33,10). The speed-up under forward load is predicted by simple pathway models that invoke a single load-dependent transition with Boltzmann-type behavior (33) and also by discrete-state stochastic models with three-dimensional energy landscapes (36).

If the application of forward load simply pulled the trailing head in front of the leading head and caused the neck linker to dock or undergo some other conformational change (one possible version of the Rice et al. (32) scenario; see also Carter and Cross, (10)), then we might not expect to see any speed-up in velocity at limiting [ATP], which would disfavor this docking. This is food for thought.

WHEN STEPPING PROCESSIVELY, DOES KINESIN SPEND MOST OF ITS TIME IN A TWO-HEADS BOUND STATE OR A ONE-HEAD BOUND STATE?

This is a very interesting and controversial question, and one that bears directly on mechanism. The many electron micrographic reconstructions that have been performed on kinesin and its relatives are not informative here, because they are not carried out under physiological conditions, especially with respect to the kinesin concentration. Biochemical experiments by Hackney argued that because the rear head of kinesin is competent to synthesize ATP, it must remain bound to the microtubule for most of the kinetic cycle. The experiments of Yildiz et al. (19), which found 16-nm steps for a single labeled head of the stepping dimmer, also lend strong support to a two-heads-bound model, because otherwise they would likely have observed alternating steps of two different values that add up to 16 nm, instead. This is because if one head stays unbound during a significant fraction of the cycle time, it will not be located directly above its microtubule binding site, but instead at a position much closer to its partner head: this motion will produce a positional offset that will affect every other step. As discussed in Yildiz et al. (38,19), attaching the fluorophore dye to a position close to the common stalk can also introduce an offset leading to alternation in the apparent step size: this is a similar geometric phenomenon. Head detachment experiments performed by the Ishiwata group, however, suggest that only a single head may be bound while kinesin is in the ADP nucleotide state (39). The addition of AMP-PNP (assumed to act as an ATP analog) forces kinesin into a state characterized by twice the unbinding force and twice the elastic modulus (40). However, it seems possible that one head may be weakly bound whereas the other is strongly bound; nevertheless, both heads remain attached to their microtubule binding sites through most of the cycle under normal stepping conditions. The recent model advanced by Carter and Cross (10), however, has kinesin bound instead by a single ''holdfast'' head, whereas its partner head remains predominantly unloaded and is free to explore the energy landscape via diffusion. This picture was supported by their observation that there was little change found in the positional variance throughout the stepping cycle (although there are several alternative explanations for this that are consistent with two heads bound). On its face, however, the current (Carter and Cross) (10) model is not easily reconciled with the data of Yildiz et al. (19). So what's bound: one head, or both? Could it be that only one head is tightly bound whereas its partner remains loosely bound throughout most of the cycle?

IS THE HEAD-NECK LINKER DOCKING MODEL CORRECT (AND DOES IT SUFFICE TO EXPLAIN ACTUAL STEPPING)? DOES KINESIN UNDERTAKE A CONFORMATIONAL ''POWER STROKE'', OR SOMETHING LIKE IT (AND IF SO, HOW LARGE IS IT)?

The neck linker docking model of Rice et al. (32) was developed on the basis of structural and EPR data obtained with kinesin monomers, and it successfully explains a great deal about kinesin's structural states on microtubules in the ADP- and ATP-bound states (as implied by nucleotide analogs intended to mimic these states). A largely qualitative model for the stepping cycle of the kinesin dimer was developed directly from these data. Critical analysis of the neck-linker docking model can be found in a review by Schief and Howard (41). Two of the more salient criticisms, which I and others have also discussed (42), are these. First, the kinesin neck linker region is only ~11–13 amino acids long, and is therefore is unlikely to generate a physical displacement of even so much as 2 nm (depending on the shape of the polypeptide chain), which compares rather unfavorably with the size of the kinesin step

at 8 nm. This shortfall is all the more dramatic when one considers that the asymmetric HoH model requires that each head domain move through 16 nm to produce the 8-nm molecular step, during which only one of the two neck linkers becomes docked. A second criticism arises from subsequent work by Rice et al. (43) that estimated the free energy associated with neck-linker docking, and found it to be just ~3 kJ/mol (note that k_BT is 2.6 kJ/mol), which is very weakly favorable from a thermodynamic perspective, and represents only a minute fraction (~5%) of the free energy released through ATP hydrolysis (50–60 kJ/mol, or ~20 k_BT). Kinesin is known to be at least 50% efficient (44), so this is an unsatisfactory result. Further free energy may be recovered during the complete kinesin cycle via other energy-release mechanisms, in principle, notably through microtubule binding, but it seems implausible that kinesin could move in any sustained way against ~6 pN loads (as it does) when that would require (2 nm × 6 pN) = 12 pN nm (~3 k_BT) of free energy per step from the docking component. Hackney (35) has argued, on the basis of a series of oxygen isotope exchange experiments, that the free energy released during the ATP binding step for a kinesin head on a microtubule is substantially larger than k_BT, at ~34 kJ/mol (13 k_BT); he attributed the low energy values obtained by Rice et al. to their use of AMP-PNP, a nonhydrolyzable analog, instead of ATP. However, these purely energetic considerations do not establish whether any of the free energy released may actually be communicated to the neck linker region through docking, or power other changes. The issue remains open.

Because Carter and Cross (10) found that ATP was required for load-induced backsteps, they proposed that rearward steps may involve some sort of head undocking, in which case ATP may actually serve to undock the neck linker, that is, exactly contrary to the original proposal of Rice et al. (32). An alternative explanation, based on the findings of Guydosh and Block (8), would be that the neck linker is unable to dock when the leading head is strained, either through the application of external load or through the internal strain created by an attached trailing head.

The Yanagida group has advocated an entirely Brownian-ratchet based mechanism, where entropy rectifies the kinesin steps (45). Based on measurements of the temperature-dependence of forward and rearward stepping rates, they found that the binding of the ''free'' head in the leading position (for a forward step) was entropically favored over binding to the trailing position (for a backward step) by ~4 k_BT. Added to the ~1–2 k_BT thought to represent neck-linker docking (but recall the caveats above), this could provide roughly 6 k_BT of energetic bias to power asymmetric, unidirectional motion.

Higuchi's group has reported one controversial experiment that purported to measure the displacement associated with power-generating portion of the kinesin cycle (46), by scoring the binding displacement toward the microtubule plus-end for beads coated with monomeric kinesin. In this fashion, they obtained an apparent ''stroke size'' of 3.5 nm, which they associated with the kinesin head. Unfortunately, however, their results cannot be considered definitive, because they may equally well be interpreted as arising from a binding artifact, coming from the changing experimental geometry during the binding event, which can induce a small movement of a kinesin-attached bead that depends on the radius of the bead, the length of the kinesin stalk, etc. To address this alternative explanation, it would be necessary to show that the 3.5 nm displacement was robust, and independent of bead size and kinesin length. Furthermore, their results (if not a binding artifact) are more consistent with the ''step'' being coupled to ADP release than to ATP binding, which seems troubling.

SO, DOES KINESIN MOVE BY A POWER STROKE OR BY A BROWNIAN RATCHET MECHANISM?

The answer is, ''Yes!'' It's important to realize that these two seemingly-different mechanisms are not mutually exclusive, so this question poses a false dichotomy. Furthermore, reaction pathways, particularly those that pass through one or more energetically unfavorable transition states on their way to an energetically favored minimum—and that constitutes the vast majority of all enzymatic reactions—require additional energy, which they transiently ''borrow'' from the thermal bath to proceed at a finite rate, according to the usual Kramers/Eyring/Arrhenius rate picture. So, in a narrow sense, an awful lot of biochemical reactions might reasonably be construed as ''Brownian ratchets''. One therefore has to be exceedingly careful about definitions when discussing these candidate mechanisms. Given kinesin's small head size and large step size, I and others pointed out early on that diffusion was likely to play a significant role in transporting a head from one microtubule binding site to the next (44). A better question to ask, then, might be this: ''What fraction of the overall kinesin step distance is associated with energetically-favored conformational motions (i.e., power strokes or similar) and what fraction is associated mainly with diffusion (i.e., Brownian movement, facilitated or otherwise)?'' Even here, the purists will cheerfully point out that any distinctions between these things are not as clear-cut as one might hope. If the kinesin head is displaced by some combination of thermal energy and elastic energy release (where the source of the latter can be entropic or electrostatic), which lead to a change in its shape as well as to a change in its position and/or orientation, does this qualify as a ''thermal motion'' or a ''conformational change''? Technically, it's both, and we're once again faced with a false dichotomy.

DO KINESIN MOLECULES WITH SINGLE HEADS REALLY MOVE PROCESSIVELY?

Kinesin's remarkable processivity, which produces motion in vitro that is qualitatively different from its nonprocessive cousin, muscle myosin, has long been a subject of fascination. Processivity at the single-molecule level was first demonstrated

using a microtubule-gliding surface assay by Howard et al. (2), and thereafter with an optical trap-based bead assay by Block et al. (3). In gliding assays, the diffusional tendency of a long microtubule to persist near a given plane permits a single kinesin dimer bound to the surface to step repeatedly before release, maintaining its grip on the microtubule over distances of 5 μm and more (2). By contrast, small beads (<1 μm diameter) bearing single attached kinesin dimers have an increased propensity to diffuse away, resulting in a run length (processivity distance) that's closer to 1 μm, which corresponds to $\sim$100 steps (3). Similar run lengths have been reported in single-molecule assays using fluorescently tagged kinesin dimers in the absence of beads (47,48), indicating that this figure likely represents the native processivity of conventional kinesin dimers.

In a now-classic study, Gelles and co-workers studied motility in vitro for a series of recombinant constructs based on conventional kinesin from *Drosophila*, consisting of the complete head domain followed by progressively shorter lengths of the stalk, with 448, 401, and 340 N-terminal residues, respectively (25). The two longer of these derivatives contained sufficient lengths of the stalk to dimerize in solution, and displayed continuous, processive movement along microtubules down to the single-molecule limit. The shortest construct, however, was a monomer in solution. When placed on beads, it could drive movement, but only at high concentrations of protein when multiple motors were involved; even then, the motion tended to be irregular. These results strongly implied that kinesin's processivity was linked in some fundamental way to the two-headed nature of the dimer. (Today, of course, we have deeper insights into the basis for this linkage, derived from observations of hand-over-hand motion.) Additional experiments using fluorescently labeled constructs also found that single monomers of conventional kinesin were unable to drive processive movement (47).

It came as a surprise, therefore, when Hirokawa and colleagues reported that single recombinant constructs based on an unconventional mouse kinesin, KIF1A, moved directionally along microtubules in both fluorescent and bead assays, at unloaded speeds exceeding 1 μm/s (49,50). Unlike conventional kinesin, KIF1A belongs to a class of motors (kinesin-3) that does not spontaneously dimerize via the coiled-coil stalk domain; its members therefore tend to be monomeric in solution. They described motion by these monomers as ''processive'', but it was visibly different from the persistent, unidirectional motion previously exhibited by members of the dimeric kinesin-1 family. In fact, KIF1A motion closely resembled a biased random walk (i.e., diffusion with drift) along the length of the microtubule, with largely bidirectional motions in apparent increments of 8 nm. In these assays, single-headed KIF1A motors supported only very weak resisting loads (<0.2 pN). Purely diffusive motion of motor domains weakly bound along the lengths of microtubules has been reported before, for example, for dynein poisoned by vanadate (51), and more recently for recombinant kinesin-13 molecules (MCAK), which target microtubule ends (52)—but such motion is thermally driven and unbiased. Still, it was established that microtubules could exhibit a weak affinity for motors that would allow them to move thermally in an essentially one-dimensional potential well. Interpreting their KIF1A results, Hirokawa and colleagues argued that kinesin could undertake a form of motility that was intrinsically different from that described previously: one not involving either head coordination or head-neck linker docking, but one that invoked directional binding of individual heads to microtubules, likely involving a positively charged loop in the head domain (known as the ''K-loop''), which showed a nucleotide-dependent binding affinity for the carboxy terminus of tubulin (53). Hirokawa and co-workers even went so far as to suggest that this asymmetrical binding might represent the universal mechanism that underlies the design principles of myosin, kinesin, and G-proteins (50).

However, subsequent work by the Vale lab cast some doubt on aspects of Hirokawa's proposal. Working with Unc104 kinesin from *Caenorhabditis elegans*, another monomeric member of the kinesin-3 family, Tomishige and co-workers demonstrated that motors could become dimerized under a whole variety of conditions (54). Once formed, such dimers moved eightfold faster than the motions that had been reported by the Hirokawa group, and they did so unidirectionally, in a fashion that closely resembled conventional kinesin-1. Presumably because of the affinity of the K-loop, a unique loop in the head domain found in the kinesin-3 family, for microtubules, individual Unc104 dimers displayed prodigious processivity, with run lengths averaging $\sim$9 μm. Deletion of the K-loop slashed the average run length to $\sim$1.7 μm, quite similar to conventional kinesin. Vale and colleagues therefore proposed that Unc104/Kif1A motors actually function as dimers within cells, not as monomers. Dimer formation seems quite plausible, because it can be promoted by the known interactions of the tails of kinesin-3 with lipid rafts, for which they have affinity, and by the high effective local concentrations of monomers achieved once bound to a common cargo. In this interpretation, the biased diffusion of putative monomers reported by the Hirokawa group may be attributable instead to transient dimer formation in a background of freely diffusing monomers, which would lead to a superposition of random motion and weakly processive, unidirectional stepping, a scenario that is notoriously hard to rule out experimentally. In response, the Hirokawa group has noted that clustering without dimerization also increases the speed of KIF1A, a property possibly related to its cellular function (55).

These concerns aside, it remains to be established whether some form of kinesin head motion associated with the microtubule docking event may be responsible for some (or all) of its ability to step along microtubules. Certainly, microtubule affinity is modulated during the kinesin stepping cycle, and the binding energy can be significant. It therefore seems plausible that any directionality associated with this modulation might be usefully harnessed to make motors move.

Recently, based on FRET evidence, Steve Rosenfeld's group has proposed a hybrid model for the motility of Eg5 (a kinesin-5 family member) that invokes two sequential steps, involving an ATP binding-dependent docking of the neck linker region followed by a rolling motion of the bound head on the microtubule concomitant with ATP hydrolysis (56).

HOW DOES KINESIN MANAGE TO TRACK PARALLEL TO A SINGLE PROTOFILAMENT OF THE MICROTUBULE?

No one really knows. There is excellent, longstanding evidence that kinesin tracks closely along a path parallel to that of a single protofilament (57,58), and even kinesin dimers subjected to sideways loads continue to track faithfully along protofilament paths (33). Furthermore, we now know that kinesin moves hand-over-hand as it does so. However, these experiments do not establish whether kinesin moves along a single protofilament or whether it moves astride two adjacent protofilaments (59,60,42). Recently, Yajima and Cross (61), using marked microtubules where axial rotation could be scored, reported that a torsional component of motion was imparted by kinesin monomers functioning in a multi-motor, gliding-filament assay. They presented a model where free kinesin head tends to diffuse and bind to the most proximal microtubule binding site; however, to explain their data, they needed to invoke some additional tilting or conformational shift to generate sustained counterclockwise rotation. However, because the relationship between monomer and dimer stepping remains unknown, it is still unclear what all this means with respect to protofilament tracking by the dimer, which could still move along a single protofilament or sit astride a pair of these.

IS THERE ANY HOPE FOR EVENTUAL AGREEMENT?

All the current difficulties aside, a consensus model may possibly be emerging, if only in fits and starts. Put another way, there are several variations on a theme that are now being played, albeit with a certain dissonance and counterpoint, and that theme goes, more or less, as follows:

1. The binding of ATP to the front kinesin head in a microtubule-bound dimer releases significant energy.
2. That energetic release drives some form of conformational change, with neck linker docking representing the leading candidate for such a change. This change results in a mainly plus-end directed motion of the rear partner head through a comparatively small displacement, perhaps just 1–2 nm or thereabouts.
3. From this state, the unbound partner head, which has ADP on it, undertakes a biased diffusional search for its next forward binding site on the microtubule (with a finite probability of reaching a rearward binding site instead).
4. The heads have now swapped their relative positions, and in so doing, the centroid of the molecule has advanced by 8.2 nm along the microtubule, the tubulin dimer repeat distance. The previous two steps are both completed very rapidly, in a time $< \sim 100\ \mu$s.
5. After the partner head has reached its forward binding site, ADP is released (leaving an empty site) and this new front head binds tightly to the microtubule, thereby leading to internal strain (perhaps communicated through the neck regions, or perhaps through the microtubule). This strain tends to suppress the premature binding of ATP to the front head until the rear head had a chance to hydrolyze its own ATP and release phosphate. (Binding to the forward site may also induce additional conformations, including the possibility of motions that are not strictly parallel to the microtubule long axis.)
6. After phosphate release from the rear head (above), strain is relieved. This allows the empty front head to rebind ATP for the next step.
7. As a consequence of all of the above, the mechanochemistry of the front and rear heads of kinesin is intrinsically different, with heads swapping roles at each step, maintaining their biochemical cycles out of phase. All in all, kinesin motion is tightly coupled to ATP hydrolysis, with 1 ATP consumed per 8-nm step, which arises from the strict alternation of the two heads moving in an asymmetric, hand-over-hand fashion.

EPILOGUE

In light of all the uncertainty associated with the foregoing discussion, and the limited extent of our present knowledge, it astonishes me how often some of my colleagues have seemed ready to declare victory based on a latest insight, experimental discovery, or model, only to be humbled—or at least transiently silenced!—by the next set of experiments to be published. A great deal more remains to be discovered about motor proteins. Nature is vastly more subtle, and generally smarter, than we tend to give her credit for being.

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