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Kinesins: Processivity and Chemomechanical Coupling

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10.1
Introduction

Movement is fundamental to cellular function, and to understand the molecular basis of cellular behavior and to design strategies to treat disease, it is important to understand the motor proteins that underlie cellular motion. Kinesins are molecular motors that use the energy derived from ATP hydrolysis to transport organelles and vesicles along intracellular microtubules. Conventional kinesin, an axonal transport motor, was the first kinesin to be identified and has been the most intensively studied. Subsequently, a number of other related motors have been identified by sequence homology to the kinesin motor domain; this kinesin family of motor proteins (often termed kinesin-like proteins, kinesin family proteins or unconventional kinesins) numbers 45 in the human genome (Kim and Endow, 2000, Miki et al., 2001) and includes motors that transport vesicles, organelles, protein complexes and chromosomes.

A primary goal of research on kinesins and other motor proteins is to understand the transduction of chemical energy into mechanical work at the level of single protein molecules. These investigations require a range of techniques including mechanical measurements on individual motor molecules, enzyme kinetic studies, structural biology, and theoretical modeling of motor mechanisms. In the last decade and a half a considerable research effort by laboratories around the world has brought forth an outline of the mechanism of kinesin mechanochemistry and provided insights into the cellular role of various kinesin family members. This chapter will describe the experiments that underlie our current understanding of kinesin chemomechanical coupling and processivity and point to the unresolved questions that drive current and future research in this area.

Conventional kinesin's processive behavior, the motor's ability to walk many steps along a microtubule without dissociating, is well established. This property is important for long distance transport as in the movement of vesicles along axons in nerve cells. Many of the kinetic steps by which kinesin transduces ATP hydrolysis into mechanical motion are understood, although there are still a num-

ber of questions regarding the precise biochemical transitions that lead to force production and uncertainties regarding the critical steps that underlie processivity. The body of knowledge on conventional kinesin has set the paradigm to which the mechanism of other kinesin family members, are compared. Studies on these non-conventional kinesins show that, while the fundamental steps in the chemomechanical cycle appear to be conserved, there are significant differences in chemomechanical coupling and processivity that adapt these motors to their diverse cellular functions.

This chapter begins by describing some of the key experiments on the motility, processivity and biochemistry of conventional kinesin. These findings put constraints on models of chemomechanical coupling, eliminating many possible mechanisms. Next, experiments investigating the nature of coordination between the two heads of kinesin are described, which lead to a model of the ATP hydrolysis cycle for an individual kinesin head. Structural studies, which provide key clues for defining a kinetic model for two-headed kinesin, are then described followed by a discussion of the current model for the two-headed kinesin chemomechanical cycle that accounts for coupling ATP hydrolysis to movement and accounts for the processive behavior of dimeric kinesin. Finally, the conventional kinesin mechanism is compared to that of two kinesin family members: Ncd, a non-processive dimeric motor and KIF1A, a processive monomeric motor.

10.2
Kinesin Motility and Processivity

The first investigations into kinesin motility utilized the *in vitro* gliding assay (Fig. 10.1), in which motors are adsorbed to a glass surface and the movement of microtubules is observed by video-enhanced differential interference or fluorescence microscopy (Allen et al., 1981, 1985) An important insight into the mechanism of kinesin motility was the observation that kinesin is a processive motor. Processivity is defined as a motor's ability to move multiple steps along its track without dissociating. The evidence for kinesin processivity came from dilution experiments in which a reduction in the motor surface density resulted in a proportional decrease in the rate that microtubules landed on the surface and started to move (Howard et al., 1989). At the lowest motor concentrations, microtubules were observed swiveling around nodal points on the surface and detaching from the surface when their trailing end moved over these points. This proportionality between activity and motor density coupled with the swiveling behavior demonstrated that individual kinesin motors could move multiple steps along microtubules without detaching. This processive movement of conventional kinesin is very different from skeletal muscle myosin motility assays where multiple motors are required to move an actin filament (Uyeda et al., 1990), and is consistent with kinesin's role as a long distance transport motor.

A second motility assay uses micron-scale glass or polystyrene beads, to which motors are attached, to visualize the movement of individual or groups of motors

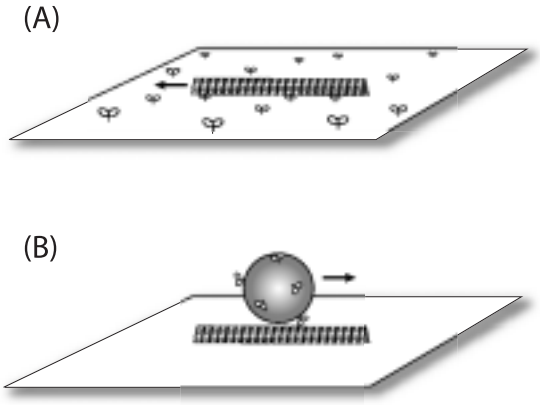

Figure 10.1. *In vitro* motility assays used to study kinesin motility. (A) Top panel shows a microtubule gliding assay in which motors are adsorbed to a glass surface via the motor tail or other domain, and microtubules are observed gliding over the surface of motors. Motor surface density can be varied as can solution conditions to test the nucleotide dependence of movement. (B) Bottom panel shows a bead assay in which microtubules are immobilized on a surface and motors are adsorbed to glass or polystyrene beads.

along immobilized microtubules or axonemes (Block et al., 1990). By decreasing the motor-to-bead ratio such that each bead has one or fewer motors attached, the bead assay confirmed that individual kinesin molecules can move processively along microtubules, and gave a more precise estimate of the distance moved per encounter of a motor with a microtubule. In the bead assay, the run length was found to be approximately 1 μm (Block et al., 1990), providing bounds for the off-rate of kinesin from microtubules. Processivity was confirmed by bead tracking experiments (Coppin et al., 1997, Kojima et al., 1997, Svoboda et al., 1993), which show that kinesin takes up to 100 8-nm steps along the microtubule before dissociating (Fig. 10.2A). This stepping behavior will be discussed further below.

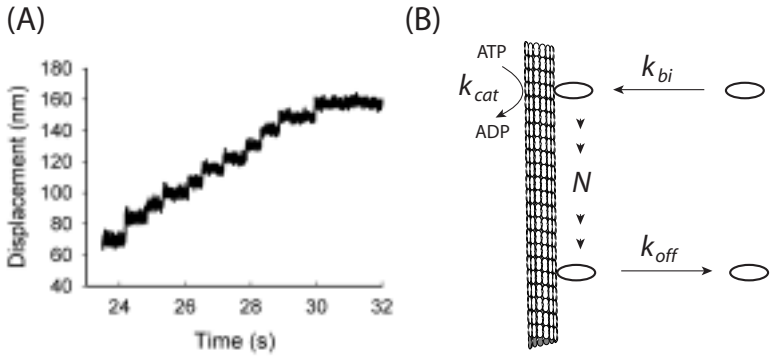

Figure 10.2. Demonstration of mechanical and chemical processivity. (A) Kinesin stepping along a microtubule. A single kinesin motor was adsorbed to a glass bead. The bead was held in an optical trap at a low ATP concentration and its position was detected using a quadrant photodiode detector. The motor takes many 8-nm steps along the microtubule without falling off: the number of steps defines the extent of mechanical processivity. **Data courtesy of Nick Carter and Rob Cross**. (B) Evidence for chemical processivity. The motor binds to the microtubule with an on-rate equal to k_{bi} and hydrolyzes *N* ATP at a turnover rate k_{cat} before detaching with a rate k_{off}. The number of ATP hydrolyzed per encounter, *N*, defines the extent of chemical processivity.

10.3
Biochemical Evidence for Kinesin Processivity

Processive motility by conventional kinesin was supported by biochemical experiments demonstrating that dimeric kinesin hydrolyzes many ATPs per encounter with a microtubule (Hackney, 1995a). The initial evidence for this chemical processivity came from ATPase measurements in which the maximum microtubule-stimulated ATPase rate, k_{cat}, was quite high (76 s^{-1} for the two-headed molecule) while the concentration of microtubules necessary for half-maximal activation, K_M, was low (240 nM (Hackney, 1995a) to 30 nM (Hackney, 1994b), depending on buffer conditions and motor concentration), indicating that dimeric kinesin motors have a high affinity for their microtubule tracks. These data implied that if motors detached from microtubules once per hydrolysis cycle, then the bimolecular on-rate for motors binding to microtubules ($k_{bi} = k_{cat}/K_M \sim 3 \times 10^8 - 3 \times 10^9$ M^{-1} s^{-1},) would need to be considerably faster than the calculated diffusion limited on-rate ($2 - 3 \times 10^7$ M^{-1} s^{-1}) (Hackney, 1995b). This discrepancy could be resolved if, instead of releasing one per ATP hydrolysis, the motor hydrolyzed many ATP per encounter. To calculate the number of ATP hydrolyzed per encounter, the bimolecular on-rate for motor binding to microtubules (k_{bi}) was determined by a different method that takes advantage of the fact that kinesin binds ADP tightly in the absence of microtubules (off rates ~ 0.01 s^{-1}) but releases ADP rapidly when it encounters a microtubule (Hackney, 1988). To determine k_{bi}, motors loaded with radioactive or fluorescently labeled ADP were combined with various concentrations of microtubules, and the release of bound ADP was monitored over time. Because motor binding to microtubules is the rate-limiting step, this measurement provides the true bimolecular rate constant, k_{bi} for motor binding. Dividing k_{cat}/K_M from the ATPase measurements by k_{bi} gives the number of ATP hydrolyzed per encounter, N. For dimeric conventional kinesin, the number was found to be 120 ATP per encounter (Hackney, 1995a; Fig. 10.2B). These biochemical data also provide an estimate for the rate motors detach from their microtubule tracks. Dividing the maximal ATPase rate of 76 s^{-1} per dimer by the number of ATP hydrolyzed per encounter gives an estimated detachment rate of 0.6 s^{-1}, which agrees reasonably with the duration of runs seen in the bead motility assay (Block et al., 1990).

10.4
Step Size of Kinesin and its Path along the Microtubule

An important constraint to defining the mechanism of kinesin motility was to define the path that kinesin takes as it steps along microtubules. The first experiment to define the path of kinesin took advantage of the fact that in microtubules with different numbers of protofilaments, the protofilament axis varies in relation to the microtubule axis. In some microtubules the protofilaments have a left-handed supertwist, others right-handed, while in some microtubules the protofilaments are parallel to the microtubule axis. By defining conditions that favor specific pro-

tofilament numbers and tagging microtubules to allow microtubule rotation to be visualized in the microtubule gliding assay, Ray et al. (1993) showed that kinesin follows the protofilament axis with great fidelity. This fidelity was examined another way by attaching motors to submicron-scale beads and tracking the bead position with nanometer precision along immobilized microtubules (Berliner et al., 1995). As the beads moved down microtubules the position of the bead perpendicular to the microtubule axis hardly changed, indicating that the motors moved parallel to the protofilament axis and only rarely switched protofilaments.

Because the tubulin dimers that make up a protofilament have a repeating 8-nm periodicity, or 4 nm per α or β monomer, the steps that kinesin takes along a microtubule were predicted to be a multiple of 4 nm, although other stepping mechanisms that utilize more than one protofilament could lead to different values. Using an optical tweezer and nanometer-scale positional detection of a motor-coated bead, Svoboda and coworkers observed steps of 8 nm as single kinesin motors moved a bead over an immobilized microtubule (Svoboda et al., 1993). This measurement defined the unitary mechanical event in kinesin motility and showed that the step size for conventional kinesin was independent of the ATP concentration and the load.

10.5 Kinesin Stoichiometry

A necessary first step towards understanding chemomechanical coupling is to define the coupling ratio: how many ATPs are hydrolyzed per 8-nm step that kinesin takes along a microtubule? Possible mechanisms range from those that have high fuel efficiency where each ATP hydrolysis event leads to a burst of steps to those with low fuel efficiency where numerous ATP are hydrolyzed without a concurrent mechanical step. A mechanism in which ATP hydrolysis is tightly coupled to movement predicts that one ATP is hydrolyzed per 8-nm step. Early kinesin ATPase experiments (Hackney, 1988) indicated that the motor hydrolyzes ATP much slower than expected based on the motility speed. However, it was recognized that full-length kinesin takes on a folded conformation in solution, allowing the tail to interact and presumably inhibit the ATPase rate of the heads (Hackney et al., 1992). This was confirmed by measuring the ATP hydrolysis rate of truncated kinesins (Hackney, 1994b) or full-length motors bound to glass beads (Coy et al., 1999a, 1999b). In these cases, the ATPase rate of nearly 100 ATP s^{-1} for dimeric kinesin correlates with the motility rate (800 nm s^{-1}) and measured step size (8 nm) (Coy et al., 1999b). Hence, under conditions where the mechanical load is small, kinesin is a tightly coupled motor and hydrolyzes one ATP per 8-nm step.

The stoichiometry of one ATP per step was also inferred by measuring the frequency of steps at various ATP concentrations, analyzing the distribution of dwell times between steps and comparing the data to various models of coupling (Hua et al., 1997; Schnitzer and Block, 1997). At low ATP concentrations individual steps are observed instead of bursts of steps and the rate of stepping varies linearly

with ATP concentration, ruling out models in which ATP binding leads to more than one step. Similarly, because the dwell times between steps are exponentially distributed at low ATP concentrations, mechanisms that involve multiple ATP binding per step are also discounted. In summary, both observations of stepping frequencies and correlation of ATPase rates with movement speeds together demonstrate that under low load (< 1 pN), conventional kinesin hydrolyzes one ATP per step. This puts bounds on chemomechanical coupling models described below. However, it is still an open question whether this 1 : 1 stoichiometry holds when the motor is moving against high external loads. Additionally, this stoichiometry does not necessarily apply to other motors in the kinesin family whose chemomechanical coupling mechanisms are tuned for different cellular roles.

10.6 Coordination between the Two Heads of Kinesin

While mechanical and biochemical experiments can clearly demonstrate kinesin processivity, uncovering the mechanistic basis in terms of structural and biochemical states and transitions between these states is more challenging. The eventual goal in understanding chemomechanical coupling in motor proteins is to formulate a kinetic model detailing the steps of the ATP hydrolysis cycle including rate constants governing transitions between these states and connections to structural transitions. However, uncovering these states and transitions is made more difficult by the presence of kinesin's two heads and uncertainties regarding the nature of their interactions and coordination.

By loading kinesin's two heads with labeled ADP, combining these motors with microtubules, and monitoring the rate of ADP dissociation under various conditions, Hackney (1994a) showed that the two heads of kinesin are not kinetically independent. These experiments again relied on the slow ADP release rate of microtubule-free kinesin ($\sim 0.01\ s^{-1}$) (Hackney, 1988), which is accelerated over three orders of magnitude by microtubule binding. The key result was that when motors and microtubules were combined in the absence of any free nucleotide, only half of the bound ADP was released at a fast rate and the other half released slowly, suggesting that one head can bind to the microtubule and release its nucleotide while the other is prevented from binding. If ATP is added, the remaining bound ADP is rapidly released, showing that ATP binding or hydrolysis catalyzes a transition in the microtubule-stimulated ATP hydrolysis cycle. This singly bound state, presumed to be an intermediate on the kinetic pathway for microtubule-stimulated ATP hydrolysis, demonstrates that kinesin's two heads do not operate independently.

The finding that in the presence of ATP both heads rapidly released their bound nucleotide left unresolved the question of whether ATP binding or ATP hydrolysis is necessary for nucleotide release by the second head. This question was answered by measuring the rate and extent of bound-ADP release in the presence of saturating concentrations of the non-hydrolyzable ATP analog, AMP-PNP (Ma and Taylor,

1997a). In AMP-PNP, the microtubule-stimulated release of the second bound ADP was 30 s^{-1}, significantly faster than the rate measured in either no nucleotide or saturating ADP. However, the ADP release rate is 3–12-fold slower in AMP-PNP than it is in ATP (Crevel et al., 1999, Hackney, 2002, Ma and Taylor, 1997a). Additionally, ATP hydrolysis by a bound monomer head, an analog of the bound head in a dimer, has been shown to be quite rapid ($>$200 s^{-1}, (Ma and Taylor, 1997b), 100 s^{-1} (Jiang and Hackney, 1997)) compared to the ADP release rate in the presence of AMP-PNP. Thus, it is likely that ATP hydrolysis by the bound head precedes binding and ADP release by the tethered head. These data are incorporated into the two-headed hydrolysis model developed below.

10.7
Testing Processivity with One-headed Kinesin Mutants

Although experiments on two-headed kinesin provide the framework for understanding processivity, the interdependency of the two heads invariably masks important features of the hydrolysis cycle. For this reason, a considerable push was made to investigate the motility and biochemical characteristics of various one-headed kinesin constructs. Initially, simple deletions were made to the coiled-coil and dimerization domain, and these truncation mutants tested for microtubule gliding (Stewart et al., 1993). These results suggested that, if attached to suitable artificial tails, one-headed kinesins were motile, but the motility speeds were very slow, the ATPase rates were very fast, and it appeared that the activity of the monomers depended upon exactly where in the sequence the truncation was made.

A second approach that successfully demonstrated one-headed kinesin motility was to fuse the kinesin head to a biotin-binding protein and attach these motors to streptavidin-coated beads (Berliner et al., 1995). These beads, coated with many one-headed kinesin motors, moved along microtubules but fluctuated in their lateral position significantly more than beads coated with two-headed kinesin. Importantly, when the motor surface density was decreased, no motility was observed, consistent with a lack of processivity. However, the lack of any activity at low motor numbers limited any conclusions regarding the mechanism of processivity.

In an effort to retain as much of the wild-type kinesin structure as possible, Hancock and Howard created a single-headed kinesin construct that retained the full coiled-coil domain, rod and tail, but which contained only one head domain (Hancock and Howard, 1998). At high surface densities in the microtubule gliding assay, these one-headed kinesin heterodimers exhibited robust gliding activity with speeds roughly 1/8th that of wild-type kinesin. To test whether one-headed kinesin is processive, the surface density of motors was systematically decreased and the activity, defined as the rate that microtubules land on and begin to move over the surface, was measured. When the motor surface density of wild-type kinesin was varied in this way, the fall in the microtubule landing rate was

proportional to the motor density and at the lowest motor densities observed (calculated as < 10 motors μm^{-2}), microtubules were observed pivoting over individual points on the surface, indicative of individual kinesin motors processively moving microtubules. In contrast, one-headed kinesin exhibited a steep dependence of landing rate on motor density and at the lowest motor densities no motility was observed, demonstrating that this one-headed kinesin heterodimer is not a processive motor. By fitting the landing rate curves to models of cooperative motor movement, it was estimated that a minimum of four to six one-headed kinesins are necessary to move a microtubule. These data suggest that the processivity of the kinesin dimer is due to the coordinated motion of its two heads.

An important insight into kinesin processivity came from the behavior of one-headed kinesin heterodimers under conditions where movement was not seen. At low motor densities, microtubules still bound to motors but did not move. This behavior was predicted by the hand-over-hand model – if the two heads are coordinated such that the first head does not release until the second head binds, then deleting the second head should result in a long-lived bound state by the remaining motor domain. The kinetics of one-headed kinesin detachment was analyzed in greater detail by adsorbing kinesin heterodimers to glass beads at densities of one motor per bead and observing the rate of binding and unbinding from microtubules (Hancock and Howard, 1999). As a comparison, wild-type kinesin, which takes approximately 100 steps per second, requires that each head must detach at a rate of at least 50 s^{-1} during the walking cycle. In contrast, the detachment rate of one-headed kinesin heterodimer in saturating ATP concentrations was 3 s^{-1}. Hence the second head accelerates detachment of the first head by at least an order of magnitude. This experiment demonstrates a second way in which the motion of the heads are coordinated (the first being the half-site release experiment). The slow detachment of the one-head-bound state is presumably an adaptation to increase the processivity of the kinesin dimer.

10.8
ATP Hydrolysis Cycle of One-headed Kinesin

Combining ATPase and microtubule binding data from one-headed kinesin heterodimers together with kinetic data from dimeric and truncated monomeric kinesins, it is possible to construct a model for the one-headed kinesin hydrolysis cycle. This chemomechanical model for a single kinesin head is a necessary first step towards building a model for the two-headed kinesin hydrolysis cycle, and it provides a framework in which to understand processivity. Here we will step through the kinetic model for one-headed kinesin (Fig. 10.3A) along with the evidence supporting each transition.

Kinesin heads in solution bind ADP tightly (Hackney, 1988) and microtubule binding causes rapid release of this bound nucleotide (at 50–300 s^{-1}; Crevel et al., 1999, Gilbert et al., 1998; Hackney, 2002, Ma and Taylor, 1997a) and strong attachment of the motor to the microtubule. The next step, ATP binding, is also

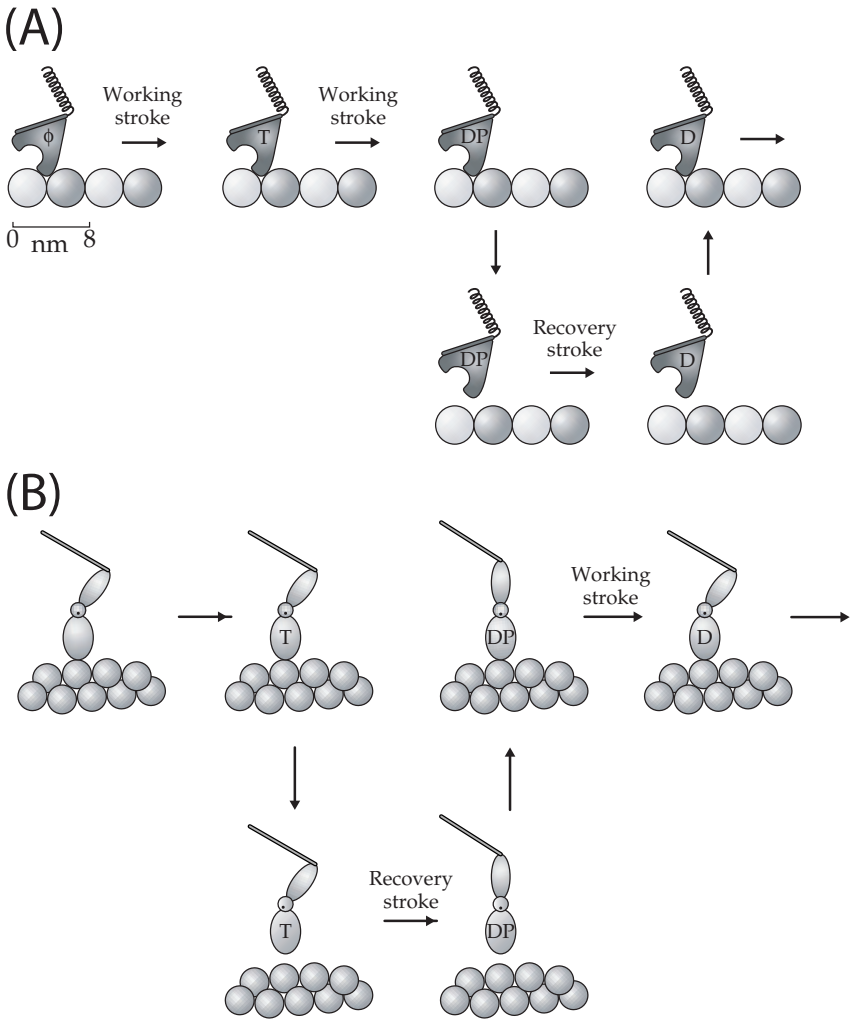

Figure 10.3. One-headed kinesin and myosin hydrolysis cycles. Kinesin binds to microtubules in the ADP state and likely detaches in the ADP-P_i state, while myosin binds in the ADP-P_i state and detaches in the ATP state. For clarity, other nucleotide states and reverse transitions are not shown.

rapid at physiological nucleotide concentrations: the bimolecular on-rate has been measured to be 0.8–3 $\mu M^{-1}\ s^{-1}$ (Gilbert et al., 1998, Hackney, 2002, Ma and Taylor, 1997b). This ATP state also binds strongly to microtubules as evidenced by the high affinity between kinesin and microtubules in the presence of the non-hydrolyzable ATP analog AMP-PNP (Lasek and Brady, 1985). The structural change underlying one-headed kinesin movement most likely occurs upon or immediately

following ATP binding, based on mechanical data from two-headed kinesin discussed below. Following ATP binding, hydrolysis occurs rapidly ($> 200\ s^{-1}$ (Ma and Taylor, 1997b), $100\ s^{-1}$ (Jiang and Hackney, 1997)) leaving the motor bound to the microtubule in the ADP-P_i state. All of the steps up to this point are generally agreed upon and consistent with kinetic studies using a range of one-headed kinesin constructs.

The detachment step for one-headed kinesin is not as broadly agreed upon, however, though the bulk of the evidence points to detachment in the ADP-P_i state and subsequent attachment in the ADP state following phosphate release. Full-length one-headed kinesin heterodimer hydrolyzes ATP at a slow rate ($\sim$ 3 ATP s^{-1}), which is similar to the detachment rate in the presence of ATP ($2.9\ s^{-1}$; Hancock and Howard, 1999). Thus, one-headed kinesin detaches once per hydrolysis cycle and both the detachment rate and the ATPase rate are slower than in the native two-headed motor. A simple explanation for these results is that one-headed kinesin detaches in the ADP-P_i state. P_i would then unbind from the detached head, completing the hydrolysis cycle. A similar model has also been proposed in which P_i release occurs when the motor is bound to the microtubule, but is immediately followed by microtubule detachment (Cross et al., 2000).

An alternate one-headed kinesin cycle, motivated by kinesin's relatively low microtubule affinity in the ADP state (Crevel et al., 1996, Romberg and Vale, 1993), holds that the motor detaches in the ADP state (Hackney, 2002, Rice et al., 1999, Vale and Milligan, 2000). However, because motors attach in the ADP state as well, the entire hydrolysis cycle would occur while kinesin is bound to the microtubule and would therefore produce no net movement. To obtain a net displacement during a hydrolysis cycle, a motor must undergo a conformational change (a power stroke) while bound to the microtubule, followed by a recovery stroke while detached. Hence, to harness movement from this cycle, kinesin–ADP would need to have two distinct biochemical and structural states – a pre-power stroke state that releases ADP rapidly upon binding to the microtubule and a post-power stroke state that releases ADP slowly and allows the head time to detach in the ADP state. There is no structural or biochemical evidence for these two states.

One apparent inconsistency in the literature is the one-headed kinesin ATPase rate. Full-length one-headed kinesin constructs display slow ATPase kinetics ($3\ s^{-1}$; Hancock and Howard, 1999), while truncated monomeric heads display hydrolysis rates as fast or faster than each head in dimeric kinesin (64 to 89 s^{-1}; Huang and Hackney, 1994, Jiang and Hackney, 1997, Moyer et al., 1996). However, the monomers display aberrant or no motility and in some cases they hydrolyze many ATP per microtubule binding event (Jiang and Hackney, 1997). The disparate models can be reconciled by positing that P_i release is slow for a microtubule-bound head, and acts as a checkpoint such that detachment from the microtubule precedes P_i release as argued above. In truncated monomer kinesins, this checkpoint is lost and P_i release followed by ADP release and subsequent ATP binding results in multiple ATP molecules being hydrolyzed without the motor moving or detaching from the microtubule.

To understand the one-headed hydrolysis cycle of conventional kinesin, it is helpful to compare it to the myosin hydrolysis cycle (Fig. 10.3B). Myosin binds to actin tightly in the ADP and no nucleotide states and binds weakly in the ATP and ADP-P_i states (Lymn and Taylor, 1971). In the standard cycle, the motor hydrolyzes ATP in the detached state, binds to actin in the ADP-P_i state, and undergoes a weak-to-strong transition coincident with P_i release. Following ADP release, ATP binding leads to rapid detachment and recovery to the pre-power stroke conformation is associated with hydrolysis (reviewed by Howard (2001)). The one-headed kinesin hydrolysis cycle presented above is similar to the myosin cycle, except that the states are shifted 90° such that attachment occurs in the ADP state and detachment occurs in the ADP-P_i state. With one possible exception (Matthies et al., 2001), all kinesin family members tested to date, share the same overall hydrolysis cycle with the ATP and nucleotide-free states binding strongly to microtubules and ADP and ADP-P_i states binding more weakly.

10.9 Structural Studies on Dimeric Kinesin

In addition to biochemical and mechanical experiments, structural studies on dimeric kinesin in solution and attached to microtubules provide the third set of constraints necessary to define a chemomechanical model for kinesin. The crystal structure of dimeric kinesin with ADP bound to both heads shows that the two heads have approximate two-fold symmetry in which the heads are related by a 120°-rotation instead of a 180°-rotation expected for exact two-fold symmetry (Kozielski et al., 1997). The heads are held together by a coiled-coil dimerization domain and the so-called neck linker that connects the core motor domain to the dimerization domain. In the crystal structure where no microtubules are present and ADP is bound to each head, the coiled-coil is closed and in each head the linker is held tightly against the core motor domain, such that a considerable rearrangement would be necessary for both heads to simultaneously bind to a microtubule.

Cryoelectron microscopic (cryoEM) reconstructions of dimeric kinesin motors attached to microtubules show that the relationship between the bound and free heads varies depending on what nucleotides are bound to each head (Fig. 10.4). In the absence of nucleotide, one head of the dimeric motor is bound to the microtubule and the tethered head is pointing to the plus-end of the microtubule and leaning to the left of the bound head (Arnal and Wade, 1998, Hirose et al., 1996). In AMP-PNP the tethered head is positioned on the right side of the bound head, suggesting that there is a conformational change following ATP binding to the bound head. This structural change is supported by experiments on monomeric kinesin in which the carboxyl terminus is labeled with a gold particle (Rice et al., 1999): in AMP-PNP the gold particle is located in the position where the tethered head is found in cryoEM reconstructions.

From ADP release experiments, it is expected that in AMP-PNP the tethered head can bind to the microtubule and release its bound ADP. Thus one might ex-

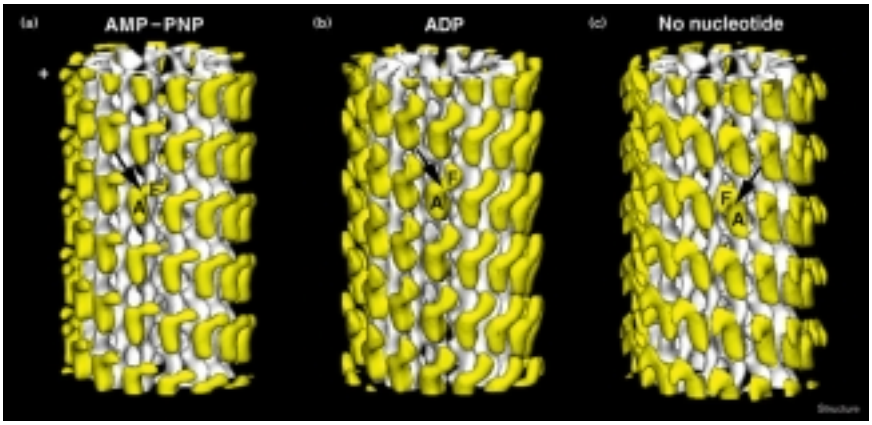

Figure 10.4. Kinesin conformational changes during the ATP hydrolysis cycle. Images are digital reconstructions from electron micrographs of frozen samples. Both heads can be seen, one attached to the microtubule (A) and one free (F). The position of the tethered head changes with nucleotide state, particularly from the nucleotide-free to AMP-PNP state, due to conformational changes in the bound head or in the interface between the two heads (arrowed). **Image by courtesy of Dick Wade, reproduced from Arnal and Wade (1998)** with permission from Cell Press.

pect that, instead of finding the second head tethered in AMP-PNP reconstructions, it would instead be bound to the next binding site along the microtubule. In fact, there is less mass attributed to the second head in cryoEM reconstructions in AMP-PNP, suggesting the second head is mobile and may be bound to the microtubule (Hoenger et al., 2000). This is one piece of evidence in support of a kinesin state in which both heads may be bound to the microtubule.

Another piece of evidence in support of a two-heads-bound conformation comes from optical tweezer measurements where motors were attached to glass beads and the rupture force and stiffness compared in no nucleotide versus AMP-PNP. In the presence of the ATP analog, the stiffness and the force required to pull the motor off of the microtubule were twice that measured in no nucleotide (Kawaguchi and Ishiwata, 2001), suggesting that both heads bind in AMP-PNP while only one can bind in the absence of nucleotide. Hence, there is both structural and mechanical evidence that when one head binds ATP, the kinesin dimer can achieve a two-head-bound state.

10.10
Two-headed Kinesin ATP Hydrolysis Cycle

Combining mechanical, biochemical and structural data for two-headed kinesin together with insights from the one-headed hydrolysis cycle, a consistent picture for the two-headed kinesin hydrolysis cycle emerges. A chemomechanical model for

conventional kinesin, originally developed by Hancock, Shief and Howard (Hancock and Howard, 1999, Schief and Howard, 2001), is presented here (Fig. 10.5) and includes some features that are broadly agreed upon and other features that are still being debated. This model provides a framework for understanding which steps are load dependent, which transitions are rate limiting, which transitions are critical for processivity, and which features of the cycle are modified in kinesin-related proteins that display novel motility characteristics.

In solution, kinesin has a high affinity for ADP and binding to a microtubule causes the release of ADP from one of the two heads. This transition (state 1) causes one head to bind tightly to the microtubule in a conformation that prevents the second head from binding. This step is supported by biochemical data discussed above (Hackney, 1995a) and by cryoEM structural studies that show that in the absence of nucleotide one head is bound to the microtubule and one head remains free (Arnal and Wade, 1998, Hirose et al., 1996). In this one-head-bound state, it is presumed that the conformation of the bound head is such

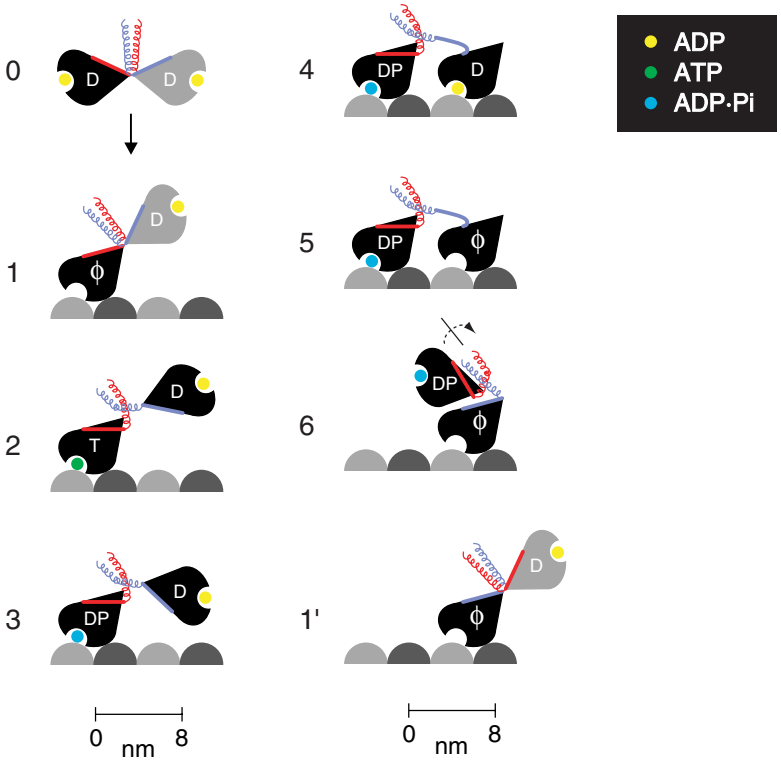

Figure 10.5. Model of the two-headed kinesin hydrolysis cycle, combining biochemical, structural and motility data. The neck-linker is shown as a coil that alternates between a docked (1) and a free conformation (e. g. 2), and a coiled-coil that alternates between a closed (1) and partially open (e. g. 2) conformation. For details see text. **Figure reprinted from Schief and Howard (2001)** with permission from Elsevier Science.

that the free head is prevented from interacting with the microtubule. Accordingly, the neck linkers for both heads are shown in their structured state, tightly associated with the core head domain.

The second step, ATP binding, occurs rapidly at physiological nucleotide concentrations (k_{on} estimated at 0.3−3 μM^{-1} s^{-1}; Gilbert et al., 1998, Hackney, 2002, Ma and Taylor, 1997b). Two bodies of evidence suggest that ATP binding induces a conformational change in the bound head that accounts for at least part of the 8-nm displacement along the microtubule during each step. As discussed above, when kinesin containing bound ADP is combined with microtubules in the presence of AMP-PNP, the release of the second ADP molecule is much faster than in the absence of any nucleotide. Although the rate is not as fast as in the presence of saturating ATP concentrations, this result nonetheless indicates that ATP binding leads to a conformational change that permits the second head to bind the microtubule and release its nucleotide.

The second piece of evidence suggesting that ATP binding induces a conformational change in the bound head comes from mechanical experiments using microneedles or optical tweezers to impose a mechanical load on a motor. At low ATP concentrations, where ATP binding to the head is the rate-limiting step in the hydrolysis cycle, the velocity of movement slows with imposed force (Meyhofer and Howard, 1995, Svoboda et al., 1993). Thus, a transition coincident or associated with ATP binding is load dependent as discussed further below.

Following ATP binding, the nucleotide is hydrolyzed to ADP + P_i and the second head rapidly attaches and releases its bound ADP locking the motor into a two-head-bound conformation (state 5). This transition is thought to involve a significant conformational change to allow the two heads to span the 8 nm between successive tubulin subunits. A restructuring of the neck linker in the forward head is a plausible explanation, but it is also possible that the linker in the bound head is stretched, or that the coiled-coil dimerization domain also partially unfolds to permit both heads to bind. In any case, the rear head is thought to be under considerable strain in this two-heads-bound state, which accelerates its detachment.

To complete the hydrolysis cycle, the rear head is detached and swung towards the plus-end of the microtubule to ready it for the next hydrolysis cycle (state 1′). Presently there is insufficient evidence to strictly define this portion of the pathway, although available evidence points to a preferred sequence. The simplest interpretation is that the strain in the two-heads-bound state (state 5) causes the rear head to detach from the microtubule and a restructuring of the neck linker and coil following P_i release swings the rear head to a forward tethered position (state 1′). It is formally possible, however, that ATP binding is rapid enough to allow ATP to bind to the forward head before the rear head is detached, followed rapidly by rear-head detachment and P_i release (Rice et al., 1999). This pathway may be preferred at high external loads, where the external force pulling backward slows the detachment of the rear head, allowing time for the forward head to bind ATP.

Another possible transition out from state 5 is for P_i to be released before rear-head detachment. Because the ADP affinity of kinesin bound to microtubules is very low (k_{off} is 50−300 s^{-1}; Gilbert et al., 1998, Hackney, 2002, Ma and Taylor,

1997a), ADP would be rapidly released, followed by ATP binding and hydrolysis without an associated step. These futile hydrolysis cycles may occur at high external loads, where the rear head detaches slowly enough to permit extra hydrolysis cycles before detachment. The 1 : 1 stoichiometry at low load confirms that they never or only rarely occur at low loads, and this is why the pathway shown in Fig. 10.5 in which rear-head detachment precedes P_i release, is preferred.

Processive kinesin motors take approximately 100 steps along a microtubule without detaching (Block et al., 1990, Hackney, 1995a), meaning that there is a 1 % probability of detachment per hydrolysis cycle. What limits kinesin processivity? Because motors bind tightly in both the ATP and nucleotide-free states (detachment rates = 0.001 s^{-1}; Hancock and Howard, 1999) it is unlikely that detachment occurs from those states or any state in which both heads are bound. Detachment most likely occurs from state 3, in which the bound head is in the ADP-P_i state and hence any mechanical load that increases the duration the motor spends in state 3 should decrease the extent of processivity. It should be noted that other models that include different intermediate states in the hydrolysis cycle predict different paths for detachment (Hackney, 2002, Yajima et al., 2002), and hence this feature of the hydrolysis cycle is not entirely resolved.

10.11 Load Dependent Transitions

A primary question in understanding kinesin mechanochemistry is: what are the structural changes that underlie the 8-nm step that occurs for each hydrolysis cycle? Is there one transition that covers the entire distance? Is the 8-nm instead made up of a number of substeps each associated with a biochemical transition? An associated question is: what transition is rate limiting at high versus low loads? The way to explore this question has been to determine which transitions in the hydrolysis cycle vary with external load.

The first evidence for a load-dependent step came from mechanical experiments using flexible microneedles or optical tweezers to impose external loads on individual kinesin motors. The speed of movement was found to decrease linearly with imposed load (Fig. 10.6) both at high ATP concentrations and importantly at low ATP concentrations as well (Meyhofer and Howard, 1995, Svoboda et al., 1993). Because ATP binding is the rate-limiting step at low ATP concentrations, it follows that small forces that slow movement must be slowing ATP binding or a step closely associated with ATP binding. If ATP binding is irreversible, this slowing could be achieved by slowing the on-rate for ATP binding. Alternatively if ATP binding is reversible, then the load could accelerate the ATP off-rate or could slow a step subsequent to ATP binding, allowing more time for ATP to dissociate and achieving the same effect of a slowed biochemical transition.

To investigate the effects of external load and ATP binding on kinesin movement in more detail, Visscher et al. (1999) developed an optical trap with feedback control whereby the sustained movements of individual motors could be observed

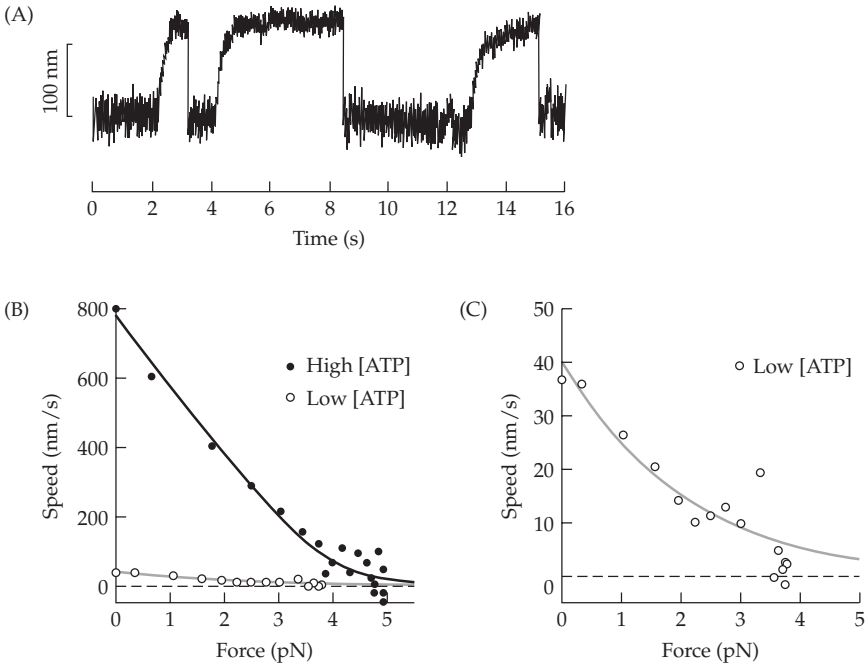

Figure 10.6. Kinesin velocity as a function of external load. A microtubule was attached to a flexible microneedle with known stiffness and the deflection of the microneedle by an individual kinesin was measured. (A) Raw data showing a number of motor-microtubule interactions in which the motor deflects the needle up to a maximal force and then eventually detaches from the microtubule. (B) Velocity as a function of force at saturating ATP concentrations (top curve) and limiting ATP concentrations (bottom curve). (C) Expanded view of data at limiting ATP concentrations showing that the velocity decreases with load even at limiting ATP concentrations. **Data reprinted from Meyhofer and Howard (1995)**. Copyright 1995, National Academy of Sciences, USA.

under constant external load. It was observed that at high loads that slowed the motor stepping rate, the ATP concentration necessary to achieve half-maximal velocity increased. Again, the data were interpreted as an external load either decreasing the on-rate for ATP binding or otherwise altering the rate constant of a step closely associated with ATP binding. The data were described by a model of ATP binding to the microtubule-bound head, the subsequent binding of the tethered head to the microtubule and the eventual detachment of the first head (Schnitzer et al., 2000). To account for the ATP dependence, a composite state was invoked in which the tethered head undergoes a 4-nm displacement from a rear to forward position and the two positions exist in a rapid equilibrium such that the external load affects the probability that the tethered head will be in the forward position according to Boltzman statistics. The result of this composite state is that by mass action there is a greater probability of ATP unbinding at high loads, account-

ing for the apparent drop in ATP affinity, while at low loads binding of the tethered head to the next binding site is favored.

Thus, the presence of one load-dependent transition coincident with or immediately following ATP binding is supported by biochemical, structural and quantitative modeling. However, the force–velocity curve at saturating ATP concentrations, which is consistently found to be linear (Coppin et al., 1997, Kojima et al., 1997, Meyhofer and Howard, 1995, Svoboda et al., 1993) or concave down (Visscher et al., 1999), implies there is at least one more force-dependent conformation (Keller and Bustamante, 2000). One candidate for a second load-dependent transition is a structural rearrangement following unbinding of the rear head (state 6 in Fig. 10.5). Because the dimeric motor is thought to be highly strained when both heads are bound, release of the rear head should pull the dimerization domain forward and an external load pulling towards the minus end of the microtubule should slow the detachment, while an external load in the direction of movement should accelerate it. In support of this idea, it was observed that for a motor moving against an elastic load, a force pulling the motor away from the microtubule in a perpendicular direction leads to an increase in the velocity at high loads (Gittes et al., 1996). This result suggests a perpendicular force can accelerate detachment of the rear head, which may be rate limiting at high loads. The magnitude and kinetics of other load-dependent transitions in the kinesin chemomechanical cycle are an area of ongoing investigation.

10.12
Ncd is a Non-processive Kinesin Family Member

In contrast to kinesin's processive behavior, the kinesin-related protein Ncd lacks processivity in either motility or biochemical experiments. These differences are consistent with the different cellular functions of these two motors: conventional kinesin is a long-distance transport motor while Ncd is involved in meiosis and mitosis where presumably many motors cooperate in spindle formation and maintenance (Endow et al., 1994). While the atomic structures of the kinesin and Ncd head domain are strikingly similar (Kull et al., 1996, Sablin et al., 1996), the attachment to their dimerization domains is quite different (Kozielski et al., 1997, Sablin et al., 1998), a feature that directly contributes to the opposite direction of movement compared to conventional kinesin, and which may explain the lack of processivity of Ncd as well. By comparing structural and chemomechanical models of Ncd and conventional kinesin it should be possible to identify the critical features that underlie processivity of conventional kinesin.

The first experiments that demonstrated a lack of Ncd processivity measured the dependence of the landing rate on the surface motor density similar to the approach taken for one-headed kinesin described above (deCastro et al., 1999). Due to the lack of a comparable control motor to confirm that the dimeric molecule binds to the surface in a functional way, the landing rate in ATP was compared to that in AMP-PNP, which promotes strong binding to the microtubule. Consis-

tent with individual Ncd motors being sufficient to bind microtubules to the surface, in AMP-PNP the landing rate was found to be proportional to the surface motor density. This control experiment confirmed that motors were bound to the surface in an active conformation and it defined the motor dilution necessary for single motor interactions with microtubules. When the experiments were repeated in ATP, much higher motor densities were required to obtain equivalent landing rates, and the landing rate fell off steeply with motor density, indicative of many motors being required to move a microtubule.

A more direct assay of the stepping behavior of Ncd utilized an optical tweezer system to bring a microtubule into contact with individual surface-adsorbed Ncd motors (deCastro et al., 2000). By attaching silica beads to both ends of a microtubule and immobilizing motors on a third bead attached to the surface of the flow chamber, the microtubule could be positioned to interact with individual motor molecules and the displacements measured directly. Instead of the stereotyped staircase of 8-nm steps seen for conventional kinesin, Ncd produced unitary steps of approximately 9 nm. To observe these steps, the duration of binding events was prolonged by decreasing the ATP concentration and multiple events were averaged to increase the signal to noise ratio. The initial binding, which was monitored by a decrease in the amplitude of fluctuations in the bead position, involved little or no displacement of the motor along the microtubule axis. However, at the end of the binding event there was a measurable displacement of roughly 9 nm along the microtubule axis followed by rapid detachment. The duration of the entire binding event varied with ATP concentration, consistent with the nucleotide-free motor holding to the microtubule and awaiting ATP binding. In contrast, the delay between the displacement and detachment, although obscured somewhat by the signal averaging, did not appear to vary with ATP concentration. These mechanical transients are consistent with ATP binding causing a conformational change in the bound Ncd head, which is followed rapidly by hydrolysis and detachment from the microtubule in the ADP-P_i state.

These mechanical events for dimeric Ncd are what might be expected from a non-processive one-headed conventional kinesin. Following binding and ADP release the motor is tightly bound to the filament, and ATP binding and hydrolysis lead to detachment (Hancock and Howard, 1999). Kinetic models of the kinesin hydrolysis cycle predict displacements upon ATP binding of 1–4 nm (Schief and Howard, 2001, Schnitzer et al., 2000), smaller than those observed from Ncd. It is possible that mechanical amplification by the Ncd dimerization domain could underlie this large observed step size.

Biochemical experiments on dimeric Ncd also point to a non-processive mechanism and highlight differences from processive kinesin. The first biochemical evidence against Ncd being processive comes from experiments where Ncd motors are first incubated with microtubules in the absence of nucleotide to promote a strongly bound Ncd–microtubule complex. When this complex is rapidly mixed with ATP, there is an initial burst of hydrolysis that has an amplitude equal to the concentration of motors present, and which is followed by a slower steady state rate (Foster and Gilbert, 2000). This stoichiometric burst is consistent with

microtubule-bound motors binding and hydrolyzing one molecule of ATP and then detaching from the microtubule, with later hydrolysis events requiring the motor to reattach to the microtubule. The second piece of evidence is that the detachment of Ncd from microtubules following rapid mixing with ATP has a fast rate of 13 s^{-1} (Foster and Gilbert, 2000), considerably faster than the overall hydrolysis rate of 2 s^{-1} (Foster et al., 1998). Finally, equilibrium binding experiments showed that in ADP plus added P_i the microtubule affinity of Ncd is lower than in ADP alone (Foster et al., 1998), suggesting that the motor detaches in the ADP-P_i state and then rapidly releases its P_i. This detachment step agrees with much of the conventional kinesin data (Hancock and Howard, 1999, Rosenfeld et al., 1996) although in both cases direct demonstration of detachment in the ADP-P_i state has proven difficult. In summary, the key feature of the Ncd hydrolysis cycle that prevents processivity is that following ATP hydrolysis, motor detachment from the microtubule (most likely in the ADP-P_i state) occurs at a faster rate than attachment of the second head.

To prevent diffusion of the motor away from the microtubule, most models of alternating hand-over-hand processivity require that during some portion of the walking cycle both heads must bind simultaneously to the microtubule. In conventional kinesin a structural transition in the neck region, melting from a β sheet conformation to a random coil or opening of a coiled-coil, is thought to provide the flexibility needed to allow both heads to bind simultaneously to a microtubule (Kozielski et al., 1997). Available structural evidence for Ncd suggests that the two heads are more tightly associated with each other than are the two heads of conventional kinesin, and thus may not be able to simultaneously bind to a microtubule. First, in the crystal structure of two headed Ncd (Sablin et al., 1998), the heads possess two-fold symmetry and are held together tightly against the coiled-coil dimerization domain by multiple associations between residues in the core motor domain and those in the coiled-coil. Second, the neck-linker that joins the catalytic core to the neck-coil is considerably shorter for Ncd (Endow and Waligora, 1998, Sablin et al., 1998), which would necessitate a considerable uncoiling of the coiled-coil neck domain to allow the tethered head to bind to the next microtubule binding site. Third, in cryoEM reconstructions of dimeric Ncd bound to microtubules (Arnal et al., 1996, Sosa et al., 1997), which show the tethered head pointing towards the minus end of the microtubule, the tethered head is considerably more well-defined than for conventional kinesin and the boundaries of the tethered head are clearly delineated, indicating that it is relatively immobile. Hence, all available structural evidence suggests that the two Ncd heads remain tightly associated which would prevent both heads from simultaneously binding to a microtubule and preclude processive movement.

10.13
A Processive Monomeric Kinesin, KIF1A

Because the prevailing model for conventional kinesin processivity involves coordination between the two heads, it came as quite a surprise when a monomeric kinesin, KIF1A, was shown to be processive (Okada and Hirokawa, 1999, 2000). KIF1A, which is thought to be involved in vesicle transport in neurons, has been shown by hydrodynamic analysis to be monomeric in solution (Okada et al., 1995). Motility of individual KIF1A molecules was observed by fusing the KIF1A head and conventional kinesin neck linker to green fluorescent protein and observing individual motors moving along immobilized microtubules (Okada and Hirokawa, 1999). The motors bound microtubules for long durations and diffused back and forth with a net movement towards the microtubule plus-end such that over time the motors accumulated at one end of the microtubules.

The key to KIF1A processivity is that the motor domain has a second microtubule binding site consisting of a loop of positively charged residues. This 'K-loop' named because of its numerous lysine residues, is thought to interact with the negatively charged carboxyl tail of tubulin, termed the 'E-hook' because it contains numerous glutamate residues. A body of evidence supports the notion that the K-loop provides a tether that holds the motor on the microtubule during the detachment phase of the hydrolysis cycle. First, when the K-loop of KIF1A or the E-hook of tubulin was deleted, the processive behavior was abolished. Second, mutants in which some of the lysine residues were deleted showed a graded decrease in microtubule affinity with deletion of lysines. Third, if the K-loop was inserted into the homologous sequence of a conventional kinesin monomer, this normally non-processive head displayed processive movement with positional fluctuations in both directions but a clear net displacement over time. Fourth, the cryoEM reconstruction of KIF1A bound to a microtubule, aided by alignment with the crystal structure of the conventional kinesin head, shows an interaction between the K-loop and E-hook (Kikkawa et al., 2000).

The motility of KIF1A is very different from the precise stepping of conventional kinesin. KIF1A makes rapid runs with amplitudes of hundreds of nanometers in both directions and periodically pauses for hundreds of milliseconds or more. This movement can be quantitatively described as one-dimensional diffusion superimposed on a steady directed component. The proposed mechanism involves diffusional events where the motor is loosely associated to the microtubule via its K-loop, together with a conventional kinesin power stroke to bias the diffusion towards the microtubule plus-end (Okada and Hirokawa, 2000). Consistent with this, when movement was analyzed in the presence of ADP only, the diffusive component continued, but the net movement towards the plus-end was abolished.

Biochemical experiments (Okada and Hirokawa, 2000) provide insight into the walking mechanism of KIF1A. The microtubule-stimulated ATPase rate of KIF1A is 110 s^{-1} and when compared to the 140 nm s^{-1} net velocity towards the microtubule plus-end, indicates that the motor most likely takes many futile steps that result in ATP hydrolysis but no net displacement. To determine the ex-

tent of chemical processivity (the number of ATPs hydrolyzed per step), the approach developed for conventional kinesin was employed. By comparing the predicted motor-microtubule association rate (k_{bi}) from ATPase measurements to the actual encounter rate measured by release of labeled ADP following microtubule binding, it was determined that KIF1A hydrolyzes nearly 700 ATP per encounter with a microtubule. Thus KIF1A is five-fold more chemically processive than conventional kinesin. Finally, the time constant for motor dissociation from the microtubule (6.3 s) calculated from the kinetic data agreed well with the detachment time constant measured from the motility assays. Linking the biochemistry with the observed diffusive motility indicates that ATP hydrolysis and movement are only weakly coupled in KIF1A.

In addition to KIF1A motility, processive movement has also been reported for a monomeric kinesin construct derived from conventional kinesin (Inoue et al., 2001). When monomeric kinesin heads were fluorescently labeled and observed in a low ionic strength motility buffer, Inoue et al. observed microtubule binding and short-distance movement along microtubules (mean displacement 40 nm), suggesting these truncated heads work by a similar processive mechanism as KIF1A. When a fusion protein was constructed with a protein that normally binds weakly to microtubules, the run lengths were increased, consistent with adding a second nonspecific binding site to the motor. The high ATPase rates and diffusive character of the movement suggests a similar loose coupling between ATP hydrolysis and movement as seen in KIF1A. However, these results directly conflict with observations made on almost identical one-headed kinesin constructs from a number of laboratories where no processive motility was observed (Berliner et al., 1995, Hancock and Howard, 1998, Okada and Hirokawa, 1999, Pierce et al., 1999, Vale et al., 1996, Young et al., 1998). At present, the differences in experimental technique and data analysis that underlie these conflicting results are yet to be reconciled.

KIF1A processivity clearly demonstrates that there are other mechanisms besides the hand-over-hand model to achieve processivity. It also sheds light onto the question of whether conventional kinesin motility requires a concerted conformational change in the head domains or a diffusional mechanism where the free head can find its next binding site. An outstanding question is whether a one-headed processive motor can move against an external force. During the diffusive phases of the motility cycle, the motor would be expected to slip backwards against a load. Experiments using individual KIF1A motors bound to beads and held in an optical trap should shed light on this question. One possibility is that during vesicle transport *in vivo* multiple motors are working together such that the probability of backwards diffusion and/or backwards slipping under load is greatly diminished.

An important caveat to the KIF1A work is that other laboratories have failed to detect processive movement by homologous monomeric kinesins. Using a similar single molecule fluorescence assay, Pierce et al. (1999) failed to detect processive movement in UNC104, a KIF1A homolog from *C. elegans*, and Rogers et al. (2001) failed to detect processive movement in KIF1D. One possibility is that the conventional kinesin neck linker which is fused to KIF1A, contributes to processivity

possibly by acting as a second positively charged tether. In support of this idea, it was shown that duplicating the neck sequence or adding extra positively charged residues to the neck coil of conventional kinesin increased the processive run length while adding negative charges reduced processivity (Romberg et al., 1998, Thorn et al., 2000). Also, cleaving the negatively charged E-hook from tubulin was found to decrease processivity in both wild-type and mutant conventional kinesin (Thorn et al., 2000, Wang and Sheetz, 2000), consistent with the idea that a second electrostatic interaction – in addition to the normal kinesin–microtubule interface – may help to keep motors associated with microtubules during processive movement. Hence, to understand whether single motor processivity of KIF1A can actually occur in cells it will be necessary to demonstrate processive movement of a KIF1A construct that does not contain the conventional kinesin neck linker domain.

Interestingly, some myosins may use a similar electrostatic mechanism to stay attached to actin. A number of studies have suggested that, in addition to the conventional actin binding site on the myosin heavy chain, in many muscle types there is a positively charged $\sim$ 40 amino acid N-terminal extension of the myosin essential light chain (ELC), located at the base of the myosin head, that interacts with actin as well. Crosslinking studies (Sutoh, 1982) provided the first evidence for this interaction, and cryoEM reconstructions of myosin heads bound to actin filaments supported this: a region of the ELC was seen stretching roughly 8 nm across the myosin head and binding to the actin filament (Milligan et al., 1990). Furthermore, this interaction was demonstrated to have functional consequences. In myosins in which the N-terminal ELC extension was naturally absent or was mutated or deleted, the muscle shortening velocity (Sweeney, 1995) and the ATPase rate in solution were accelerated (Timson et al., 1998), while the apparent actin affinity was decreased (Timson et al., 1998). These data suggest that in some myosin isoforms this second actin binding site increases the duration that myosin is bound to actin during each hydrolysis cycle, perhaps by helping to orient the myosin head to optimize actin binding. Hence, it appears that this performance-enhancing feature, a non-specific filament binding site that complements the primary filament binding site, may be a general feature of cytoskeletal motor proteins.

10.14
Unresolved Questions

To gain further insights into kinesin chemomechanical coupling, future investigations will require the convergence of single molecule mechanical studies, biochemical investigations and quantitative modeling. There are a number of mechanical questions to be resolved. First, how do external forces alter the biochemical rates in the hydrolysis cycle? Mechanical forces can alter both forward and reverse rate constants and the magnitude of the effect will depend on the precise direction of the applied forces. The influence of forces with components orthogonal to the direction of motion should give insight into the three-dimensional conformational changes underlying movement.

A second mechanical question concerns the existence of substeps. The available biochemical and mechanical data predict there are at least two substeps in the kinesin walking mechanism. By examining the position of kinesin-coated beads stepping along a microtubules with microsecond time resolution, Nishiyama et al. (2001) recently found evidence for two 4-nm substeps in each 8-nm kinesin step. Further resolution of these substeps will likely require mutant motors that either have structural alterations that magnify subtle movements or have altered biochemical properties that slow the transitions sufficiently to observe the substeps.

A third mechanical question concerns the overall conformational change that occurs in two-headed kinesin during its walking cycle. Because the two heads in conventional kinesin are identical peptides, it might be thought that they undergo identical conformational changes during their ATP hydrolysis cycles (a symmetric hand-over-hand mechanism). This differs from walking mechanisms analogous to left and right feet marching along a microtubule: a symmetric mechanism would mean that the two heads would rotate around one another, introducing one-half twist per step (Howard, 1996). Such twisting up of the motors is not consistent with high-density assays, which show that tens to hundreds of motors can move microtubules for many microns: if each head rotated 180° per 8 nm, an impossible number of twists would be accumulated in each motor. Even in single-molecule assays where a motor takes up to 100 steps, symmetric models would imply an unfeasible amount of torsion (50 twists), which is expected to cause the microtubules in sliding assays, to swivel in one direction during translocation across the surface. Hua et al. (2002), using surface-immobilized kinesin and low ATP levels to slow the stepping rate, carefully looked for evidence of microtubule rotation during translational movement and found no evidence of such twisting. This result implies that either the rotational strain is relieved during each step (asymmetric hand-over-hand model) or that the motor steps along in an 'inchworm' type mechanism that introduces no rotational strain in the two-headed motor.

Motors with different directionality have already provided insights into structural aspects of the kinesin power stroke and continued characterization of kinesin family motors should provide further insight into the fundamentals of chemomechanical coupling in kinesins. A corollary of this, is understanding population effects in non-processive motors – because these motors work as aggregates in cells it will be important to understand motor properties that emerge when motors work together in aggregate. These questions will require the convergence of single-molecule measurements, enzyme-kinetic studies, structural investigations and theoretical modeling. The answers gleaned from these investigations will help us to understand not only kinesin mechanochemistry, but will help define the physical and molecular principles underlying all cellular movement.

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