Title
Chemical and structural analysis of kinesin motility

Author
Romberg, Laura,

Publication Date
1997

Peer reviewed|Thesis/dissertation

Chemical and Structural Analysis of Kinesin Motility

by

Laura Romberg

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: . . . . . .

Acknowledgments

The Thesis Committee: I would like to thank Ron Vale, who has been a great example of how to use experiments to ask interesting questions, and who has spent a lot of time teaching me how to write and speak well. He has also had patience with me as I've tried to learn how to make decisions in science and in my career, and has at times been an excellent cheerleader, I thank my thesis committee, Roger Cooke and Tim Mitchison, for being extremely supportive throughout my time at UCSF. Roger provided useful discussions about my experiments, both when I was studying for my orals (and Ron was in Indonesia) and when I was planning my neck mutation project. Tim was always encouraging towards me, particularly during my orals and when I discussed with him my possible plans for the future.

The Vale Lab: I thank all the members of the Vale lab, past and present, for providing a home at UCSF. Being in the lab is like having 15-20 siblings or roommates. You spend so much time with these people that they end up knowing you too well, picking on you too much, and never cleaning up after themselves as often as you think they should.

The Early Years: Of the people who made a particular difference to my experience at UCSF, I need to start by thanking the infamous Frank McNally. During my first years at UCSF, when the lab was small and isolated, he personally taught me 50% or more of what I learned. He was (until the stress hit) always willing to explain technical details at length and to discuss projects in areas that were not his own. Despite certain "challenging" aspects of dealing with Frank, I sorely missed him when he left. I'd like to thank Jim Hartman for being a fine repository for found plastic objects, thereby keeping

said objects out of the garbage dump, a goal we all know is important to him. In addition, I thank him for our many conversations about words, and for never killing me at any point during my graduate career. I have to thank Josh Niclas for appreciating Dear Lucy Mouse (a.k.a. The Missing Link, may she Rest In Peace) and for providing the yummy trout chow which she ate so cutely.

The Bloated Lab Years: I appreciate Brinda Govindan and Dara Spatz Friedman for bringing both estrogen and enthusiasm with them when they joined the lab, two things that were sorely lacking at the time. I am also reassured to know that Dara's lentil soup exists to protect the world in case of an alien invasion. I was glad to have had Guenther as a back-to-back deskmate, and enjoyed both our conversations about his heroic telescope-building efforts and also our spontaneous discussions of kinetics. In recent times, Sarah Rice has somewhat taken over Guenther's role as a kinetics discussion partner. In addition, I have to thank Sarah for helping me with my summer project. Sarah is disappointed in herself for not having yet achieved a true Buddha nature in which she can control her core body temperature through shear force of will. However, if she had not agreed in the first place to swim from Alcatraz with me, or lent me her husband as a substitute partner, I might never have gone through with the swim, and the project contributed greatly to the maintenance of my sanity during the time I was finishing up in the lab. Finally, I need to thank Dan Pierce for all the time he spent helping me with my neck mutation experiments. Among other things, he patiently answered far too many phone calls on Saturday afternoons when the damned microscope was misaligned AGAIN.

The Friends of Microtubules: I thank the friends of microtubules for providing an incredible resource of knowledge and expertise which will be hard to find anywhere else. I am glad (for the most part) to have organized microtubule meetings, because it gave me a chance to meet everyone at a time when the Vale lab was still isolated on the 8th floor. It also gave me the opportunity to learn that the Mitchison lab, despite appearances to the contrary, is actually quite conservative, in fact almost reactionary, when it comes to changing things from The Way They've Always Been Done. On a more personal note, I have to thank Claire Walczak and Sarita Jain for being extraordinarily helpful and patient with all my questions, both during the time I was applying for post-docs, and more recently, as I've been preparing to leave.

Life Outside The Lab (Huh?): Outside the lab, many different friends have made my stay in San Francisco more enjoyable. However, here I want specifically to thank a few people whose presence and support have helped me to get through graduate school. The dependable friendly smiles and fellowship of my swimming buddies were bright spots that helped get me through a few too many bleak days. In addition, swimming has been a form of therapy for me, providing a time when I could turn off my brain, not make any decisions, and by just doing what I was told, usually achieve success. This provided a nice contrast to working in the lab. I have to thank Sean Bell for providing tons of help in my last frantic days in San Francisco. My parents have been so continuously there for me throughout my life that I usually don't consciously notice their support. However, during graduate school, they have always kept up with how things are going for me, and I have always known how much they care. My mother has cared not only how I'm

doing, but also what I'm doing, so that for various reasons throughout the years, I've described to her things such as how to do a western blot, or what FRET is, and I am proud to follow her in becoming the next Dr. Romberg. Finally, I would like to thank Curtis Carlson, who for last three years (much to the enrichment of Ma Bell) has listened to all the ups and downs of my life in the lab, and has laughed at my jokes at times when no one else would, both of which were invaluable.

Author: Laura Romberg [SMTP:romberg@socrates.ucsf.edu] at uk.magazines
Date: 03/12/97 00:50
Priority: Normal
TO: Louise Banbury at MM-4
Subject: Mail from Nature Web Service

Subscriber name: Laura Romberg

To whom it may concern,
I would like permission to include in my Ph.D. dissertation a copy of the paper cited below which was published in Nature on January 14, 1993. The dissertation will be microfilmed by University Microfilms Incorporated, and they request permission to supply single copies on demand.
Romberg, L. and Vale, R.D. 1993. Chemomechanical cycle of kinesin differs from that of myosin. Nature 361:168-170.
If you could respond by fax to (415) 476-5233 in the next couple days, I would appreciate it. If there are any problems, I can be reached by e-mail at: romberg@socrates.ucsf.edu.
Thanks very much.
Sincerely,
Laura Romberg
Department of Biochemistry
Box 0448
University of California, San Francisco
San Francisco, CA 94143-0448
Tel. (415) 476-8712
Fax (415) 476-5233
e-mail romberg@socrates.ucsf.edu

TO: LAURA ROMBERG
FROM: LOUISE BANBURY

NATURE
Permission to reprint is granted by Macmillan Magazines Limited. Porters South, Crinan Street, London N1 9XW
COPYRIGHT CREDIT LINE REQUIRED. Please follow this sample:
Reprinted with permission from Nature (reference citation)
Copyright (year) Macmillan Magazines Limited.
APPROVED BY L. Banbury DATE 3/12/97
Nature Copyright Office
☐ If this box is checked, author permission is also required.
See original article for author's.

Chemical and Structural Analysis of Kinesin Motility

by

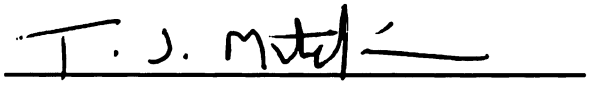
Laura Romberg

ABSTRACT

Kinesin is a cytoskeletal motor protein that transports vesicles along microtubules. Since only a few kinesin molecules are responsible for moving each vesicle, efficient transport depends on the motors remaining tightly associated with the microtubule. Kinesin is therefore processive, meaning that a single molecule can move across a microtubule for microns without releasing.

Processivity requires that kinesin remain bound to the microtubule for >99% of each ATP hydrolysis cycle. However, the motor must release in order to find the next tubulin binding site. To determine the hydrolysis intermediate during which release could occur, we probed the affinity of kinesin for microtubules in the presence of ATP analogs and inhibitors that prolonged those intermediates. In motility assays with ATPγS, single kinesin molecules remained tightly associated with the microtubule, implying that kinesin with unhydrolyzed nucleotide has a strong affinity for tubulin. In the presence of high concentrations of ADP, microtubules dissociated readily from single motors, suggesting that kinesin-ADP may be a weak binding intermediate. These strong and weak intermediates are reversed in myosin, implying that the coupling of hydrolysis to mechanical motion differs between the two motors.

To enable processivity, the two heads of kinesin dimers are thought to coordinate to move hand-over-hand along the microtubule. We predicted that a coiled-coil in the neck of the motor coordinates the heads by connecting them with a rigid structure that unwinds during parts of the mechanochemical cycle. Such unwinding would allow the heads to span the 8 nm distance between tubulin binding sites. To test these ideas, a series of kinesin mutants with alterations in the neck coiled-coil were fused to GFP. Processivity was assayed using a microscope capable of visualizing single GFP molecules. Substituting a stable coiled-coil did not greatly alter the motor's processivity, making it unlikely that extensive unwinding occurs during motility. In addition, although deleting the coiled-coil from the neck significantly reduced processivity, it did not abolish it. Surprisingly, duplicating one turn of the coiled-coil increased processivity. These results suggest that the neck coiled-coil influences processivity but is not essential to it.



Tim Mitchison, Ph.D.

Committee Chairman

Ronald Vale, Ph.D.

Advisor

Table of Contents

List of Tables

CHAPTER 1

CHAPTER 3

List of Figures

INTRODUCTION

CHAPTER 1

CHAPTER 2

CHAPTER 3

INTRODUCTION

Motor proteins establish the physical organization of the cell by driving the movement and localization of subcellular structures. Two types of filaments form the tracks along which three families of motor proteins move: actin supports myosin movement (Weiss and Leinwand, 1996), while microtubules form the tracks for kinesin and dynein (Bloom and Endow, 1995; Gibbons, 1996; Porter, 1996). Each motor family in turn consists of up to 30 different proteins specialized for particular functions in the cell. By comparing the mechanisms by which these different proteins move along filaments, the properties that are essential to motor proteins can be distinguished from those that can be tailored to serve a particular cellular purpose.

Two of the most thoroughly studied motors are skeletal muscle myosin (hereafter referred to as "myosin,") (Rayment, et al., 1996) and conventional kinesin (Howard, 1996). These proteins have a similar structural organization (Figure 1). Two catalytic domains or "heads" contain the nucleotide and filament binding sites and are dimerized by a coiled-coil stalk and linked to a tail domain that is responsible for localizing the motors to either thick filaments or membranous vesicles (Goldstein, 1993; Kuznetsov, et al., 1988; Mooseker and Cheney, 1995). The sequences of the head domains are conserved (>30%) within the kinesin and myosin superfamilies, but the tails and any associated light chains are divergent, allowing a wide variety of translocation events to be independently performed and regulated. Surprisingly, crystal structures of myosin (Rayment, et al., 1993) and kinesin (Kull, et al., 1996; Sablin, et al., 1996) motor domains have shown that although these proteins share almost no sequence homology, they are structurally related, suggesting that they may share a common ancestor, and potentially also a common mechanism of movement.

Coupling ATP hydrolysis to movement along the polymer

All motor proteins that travel unidirectionally along a polymer lattice must possess a few basic properties. They must be able to bind reversibly to sites along the length of the polymer, and in order to make any net progress, they must bias their direction of movement between these sites. These two tasks are accomplished using the energy from nucleotide hydrolysis. Motor proteins must therefore couple a chemical cycle of ATP hydrolysis to a mechanical cycle consisting of filament binding and movement. The intermediates in nucleotide hydrolysis act as allosteric affectors, causing the motor to alternate between states that have strong and weak polymer affinities, and between conformations that alter the position of the motor relative to the polymer. Because the nucleotide intermediates appear in a fixed order during the hydrolysis cycle, conformational changes in the motor also occur in a fixed sequence. The motor moves forward when it has bound to the polymer and reverts to its original conformation only after it has released, thereby producing directional movement.

The mechanochemical cycle of myosin is best understood (Hackney, 1996). In the absence of nucleotide, myosin binds strongly to actin. Binding of ATP to this acto-myosin complex is highly favorable, and the energy from nucleotide binding is used to release the motor from the filament. Nucleotide hydrolysis occurs off the filament and without a large change in the free energy of the system. As a result, the motor is now spring loaded. Rebinding of the myosin to actin catalyzes P_i release, which in the absence of actin would be quite slow. Following P_i release, conformational changes in the motor bind it tightly to the filament and produce the powerstroke that

drives actin sliding. In the last step of the cycle, ADP is released, and the motor remains attached to the filament ready to bind another ATP.

Initial experiments suggested that kinesin's chemomechanical cycle was similar to that of myosin. Like myosin, kinesin binds tightly to its filament in the absence of nucleotide, but releases upon addition of ATP (Vale, et al., 1985). In addition, kinesin was found to hydrolyze ATP extremely slowly in the absence of microtubules (Kuznetsov and Gelfand, 1986). The microtubules catalyze the release of ADP (Hackney, 1988; Hackney, et al., 1989) in a manner analogous to the acceleration of P_i release by actin. By having a step in the hydrolysis cycle accelerated by the presence of the polymer, the motor does not waste ATP when it is not translocating. These similarities between the two motors suggested that kinesin's mechanochemistry might parallel that of myosin in many other details (Hackney, 1992). However, even given the similarities described above, the sources of energy from ATP hydrolysis (nucleotide binding and P_i release following hydrolysis) could be coupled in many different ways to the energy requiring mechanical steps (release of the motor from the polymer and directional movement).

To understand kinesin's mechanical cycle better, I examined how microtubule binding affinities change throughout the hydrolysis cycle (Romberg and Vale, 1993, details in chapter 1). Particular intermediates in the hydrolysis cycle were extended by the addition of nucleotides or nucleotide analogs which mimicked those intermediates. The affinity of kinesin for the microtubule was assayed by observing microtubules being transported by single kinesin molecules across a microscope slide surface and measuring the time the motor remained bound to the microtubule under each nucleotide condition. The results indicated that unlike myosin, kinesin remains

attached to the polymer immediately following ATP binding and releases when ADP is in the active site.

More recent studies have furthered our understanding of kinesin's properties during each intermediate of the ATPase cycle. Both kinetic and equilibrium binding experiments have confirmed that kinesin does not detach from the microtubule due to ATP binding. Instead, kinesin has a weak affinity for the microtubule when it is bound to ADP (Crevel, et al., 1996; Ma and Taylor, 1995). Although the timing of force production in the ATPase cycle has not yet been determined, like for myosin, it probably occurs sometime after nucleotide hydrolysis (Higuchi, et al., 1997). These results have confirmed that both similarities and differences exist between kinesin and myosin in their coupling of ATP hydrolysis to the mechanics of motility.

Processivity and the kinesin motor

Adaptations of myosin and kinesin to their cellular environment

Different motor proteins are tailored to work in different environments. For example, there is a dramatic variation in the number of motor molecules involved in moving a cellular structure. This value may differ by as much as 5 orders of magnitude between muscle fibers contracting due to myosin and vesicles transported by kinesin (Ashkin, et al., 1990; Cooke, 1997; Miller and Lasek, 1985). Many of the properties of these two motors can be attributed to the conditions under which they must work.

Myosin is arrayed in an almost crystalline structure with thousands of motors contributing to the sliding of each actin filament. During muscle contraction, after finishing a powerstroke each myosin rapidly releases from the actin in order not to produce a drag on the filament that would inhibit the

action of other motors. Thus myosin has what is termed a "low duty ratio," meaning that the motor spends a low percentage of each ATP hydrolysis cycle bound to the polymer (Cooke, et al., 1982; Uyeda, et al., 1990). This low duty ratio is possible because when an individual myosin releases from the actin, the other proteins in the sarcomere hold the structure together. The low duty ratio becomes less advantageous during isometric tension, however, when filaments are no longer rapidly sliding but instead are trying to maintain their position against a force. Motors that cannot complete their powerstroke need to remain bound to the actin in order to contribute to the tension in the muscle. Myosin's duty ratio therefore changes, so that the motor remains in the strong actin-binding ADP state for longer periods of time. This results in less ATP being hydrolyzed, a phenomenon known as the Fenn effect (Bagshaw, 1993).

In contrast to myosin, only a small number of kinesin motors work together at the surface of each vesicle (Ashkin, et al., 1990; Miller and Lasek, 1985). In order for a vesicle to be efficiently transported across long distances, each motor needs to remain tightly associated with the microtubule. To this end, a single molecule of kinesin can move for microns across a microtubule without releasing (Block, et al., 1990; Howard, et al., 1989). So far kinesin is the only cytoskeletal motor for which such a property has definitively been shown.

Models for processivity

The ability of a protein to move for long distances along a polymer, undergoing many rounds of nucleotide hydrolysis without releasing and diffusing away, is termed "processivity". The term was first defined for DNA polymerase, an enzyme that is able to polymerize 1,000's of base pairs of DNA

after a single diffusional encounter with a template. This enzyme's processivity is due to a donut shaped subunit that acts as a sliding clamp to sterically block release from the DNA (Wyman and Botchan, 1995). However, no donut shaped subunits exist to explain processivity in kinesin.

A motor could be processive if, in contrast to myosin, it had a high duty ratio and released from the microtubule only briefly during its ATP hydrolysis cycle. There would then be little opportunity for it to diffuse away from the filament. However, since the kinesin motor core is only 7 nm across (Kull, et al., 1996), it is unlikely that a single motor domain could move the full 8 nm step between tubulin dimers (Svoboda, et al., 1993) without spending a significant time released from the polymer.

Alternatively, the motor could weakly interact with the microtubule in a way that would allow it to slide continuously along the length of the filament (Vale and Oosawa, 1990; Vale, et al., 1989). Thus, weak ionic bonds could substitute for the sliding clamp seen in DNA polymerase. Under certain circumstances, kinesin does diffuse longitudinally along microtubules, albeit only when the motor has been damaged by truncation or freeze-thawing (Stewart, et al., 1993; Pierce and Romberg, unpublished observations).

Kinesin's processivity could also be explained by a mechanism which combines aspects of the two models described above. Each tubulin dimer might possess multiple kinesin binding sites. If the motor remained bound to one site while it released from another, the chance of diffusing away would be minimized. RNA polymerase has been proposed to possess two independent DNA binding sites and to move processively along templates via just such a mechanism (Chamberlin, 1995). Indeed, recent reports have shown that the C-terminus of β tubulin contains a secondary kinesin binding

site. This region is not essential for single motor motility, but it increases both kinesin's affinity for the microtubule and the motor's processivity (Tucker and Goldstein, 1997, Sheetz, personal communication).

In all the above models, processivity arises from properties within an individual kinesin head, although dimerization of the motor would further increase the chance that the kinesin remained bound to the microtubule. However, the dimeric structure of the kinesin motor suggests a mechanism of processivity in which possessing two heads is essential. If the binding of the two heads to the microtubule alternated in a coordinated manner, the motor could move hand-over-hand along the microtubule. The Rep protein is a dimeric helicase that is thought to move along DNA in just this manner (Lohman and Bjornson, 1996).

In order to determine whether kinesin's processivity depends on its having two motor domains, I attempted to construct and purify a heterodimeric kinesin that possessed only one motor head attached to a fully dimeric neck and stalk (details in Chapter 2). The presence of the dimeric stalk was intended to preserve the native characteristics of the motor and to allow it to be attached to surfaces during motility assays. However, two-headed homodimers are formed as a side product during protein expression, and separation of these side products proved to be difficult. During the course of the purification attempts, techniques for observing the motility of truncated motors advanced, so that experiments could be performed on monomeric motors in the absence of the dimeric stalk. These monomeric kinesins were never observed to move as single molecules (Berliner, et al., 1995; Vale, et al., 1996). More recently, the heterodimer described above was successfully purified by (Hancock and Howard, 1996) and their results

confirmed that even when the normal neck and stalk structures are present, a single motor domain is not sufficient for processivity.

Mechanisms of head coordination

Coordination of kinesin's motor domains has formed the basis for two opposing models for processivity. The main difference between the models is in their predictions about the amount of time the motor spends with one versus two heads bound to the microtubule. In the first model, steric constraints prevent the two heads from binding simultaneously to the microtubule during much of the hydrolysis cycle. Thus, binding of one head prevents the binding of the other, and once the second head has contacted the microtubule, the first is pulled off. The cycles of the two heads remain asynchronous, resulting in processive movement (Hackney, 1994; Jiang and Hackney, 1997; Ma and Taylor, 1997). The second model proposes that the two heads can bind simultaneously to the microtubule, but cannot release simultaneously. Once a head has released from the microtubule, it rapidly finds a new binding site before the second head has a chance to let go (Gilbert, et al., 1995).

For kinesin to remain tightly associated with the microtubule, it seems more logical for the motor domains to bind to tubulin readily, rather than to prevent each other from binding. However, much evidence has accumulated to suggest that only one head of kinesin interacts with the microtubule at a time. Kinetic measurements have indicated that the microtubule cannot catalyze the release of ADP from both heads of the dimer simultaneously (Hackney, 1994). Cryo-electron microscope images (EM images) have supported this by showing one head of kinesin bound to the microtubule while the other is oriented perpendicular to the polymer axis (Arnal, et al.,

1996; Hirose, et al., 1996). In addition, monomeric kinesin remains bound to the microtubule through several rounds of ATP hydrolysis, suggesting that normally the presence of the second head helps to release the first from the microtubule (Jiang and Hackney, 1997).

Nonetheless, asynchrony of motor domain binding seems insufficient to allow kinesin to be processive. In addition, an intermediate with both heads bound to the microtubule would need to exist at least transiently. This need is particularly evident from experiments in which kinesin remains processive despite forces pulling the motor away from the microtubule (Coppin, et al., 1995; Svoboda, et al., 1993). Under these conditions, if both heads were released from the microtubule for more than 1 μs, the motor would be pulled away from the polymer (Howard, 1996). However, based on the relative positioning of the two heads in the crystal structure of the dimer (Mandelkow, submitted), kinesin does not appear be able to span the 8 nm between neighboring tubulin dimers. To allow simultaneous binding of the two heads, the connection between them needs to change.

At least two possibilities exist for how a change in the relative positioning of the two heads could allow for processivity. First, the structure of the connecting sequences might melt, becoming longer and more flexible. As a result, the previously constrained motor domain would become free to undergo a diffusional search for the next tubulin binding site. Alternatively, the connection between the heads could remain rigid, but the structure of the heads and neck could change and act like a lever arm to swing the second head closer to its tubulin binding site (Figure 2).

Experiments with truncated motors have mapped the region that connects the heads to the ~40 amino acid "neck" domain that is C-terminal to the motor core (Correia, et al., 1995; Huang, et al., 1994). The essential core of

the protein consists of the first ~340 residues of kinesin and forms a monomeric, non-processive motor. However, the addition of the neck to this core is sufficient to dimerize the protein and enable processivity (Berliner, et al., 1995; Vale, et al., 1996). Initially, it was not clear what structure the neck formed. The presence of heptad repeats indicated that it had the potential to form a coiled-coil, consistent with its ability to dimerize the motor. However, it appeared that many amino acids in the neck would destabilize a coiled-coil structure (Huang, et al., 1994; Tripet, et al., 1997; Young, et al., 1995). The conservation of these destabilizing amino acids led to a model in which part of the neck forms a coiled-coil that melts transiently during the chemomechanical cycle in order to allow both heads to simultaneously bind to the microtubule.

Alterations were made in the neck heptad repeats to test how stability and flexibility in this region affect processivity (details in chapter 3). Mutations were chosen that might be informative regardless of what the actual structure of the neck or mechanism of processivity might be. Within the context of a kinesin dimerized by the presence of ~130 stalk residues, the heptad repeats in the neck were either deleted, stabilized, extended, or disrupted with a flexible linker.

The results were surprising in almost every instance. It was expected that the neck formed a specific connection between the heads that was essential to processivity; instead, the coiled-coil in the neck could be deleted and the motor remained somewhat processive. In addition, stabilizing the neck to inhibit melting did not prevent processive movement. However, changing the flexibility or sequence of the coiled-coil did decrease the number of steps kinesin could take, indicating that the region has an influence on single motor motility. Unexpectedly, duplicating seven amino acids in the

coiled-coil actually increased the length of the motor's runs, suggesting that kinesin in nature has not evolved to possess optimal processivity. Inside a cell, if the dense packing of filaments and organelles in the cytoplasm blocked the path of a moving vesicle, it might need to release from the microtubule in order to continue on its way (Howard, 1996). Perhaps then the motor has evolved to balance processive movement with the ability to release from the microtubule when necessary.

Figure 1: Motor Structures

a) Conventional kinesin: i) Globular catalytic domain. This region contains the ATP and microtubule binding sequences. ii) Neck 1. A β-strand that lies parallel to the central β-sheet of the catalytic domain. It is conserved in N-terminal kinesins. iii) Neck 2. A ~35 a.a. coiled-coil is present in N-terminal kinesins and is highly conserved in conventional kinesin. iv) Hinge 1. v) Stalk 1. A non-conserved coiled-coil region. vi) Hinge 2. vii) Stalk 2. A non-conserved coiled-coil region. viii) Tail. This region binds light chains and the kinesin cargo.

b) Myosin: i) Globular catalytic domain. This region contains the ATP and actin binding sequences. ii) Neck. An ~80 amino acid α-helix bound by two light chains. This region is thought to act as a lever arm during motility. iii) Stalk and iv) Tail. Long coiled-coil regions dimerize the motor and enable assembly into thick filaments.

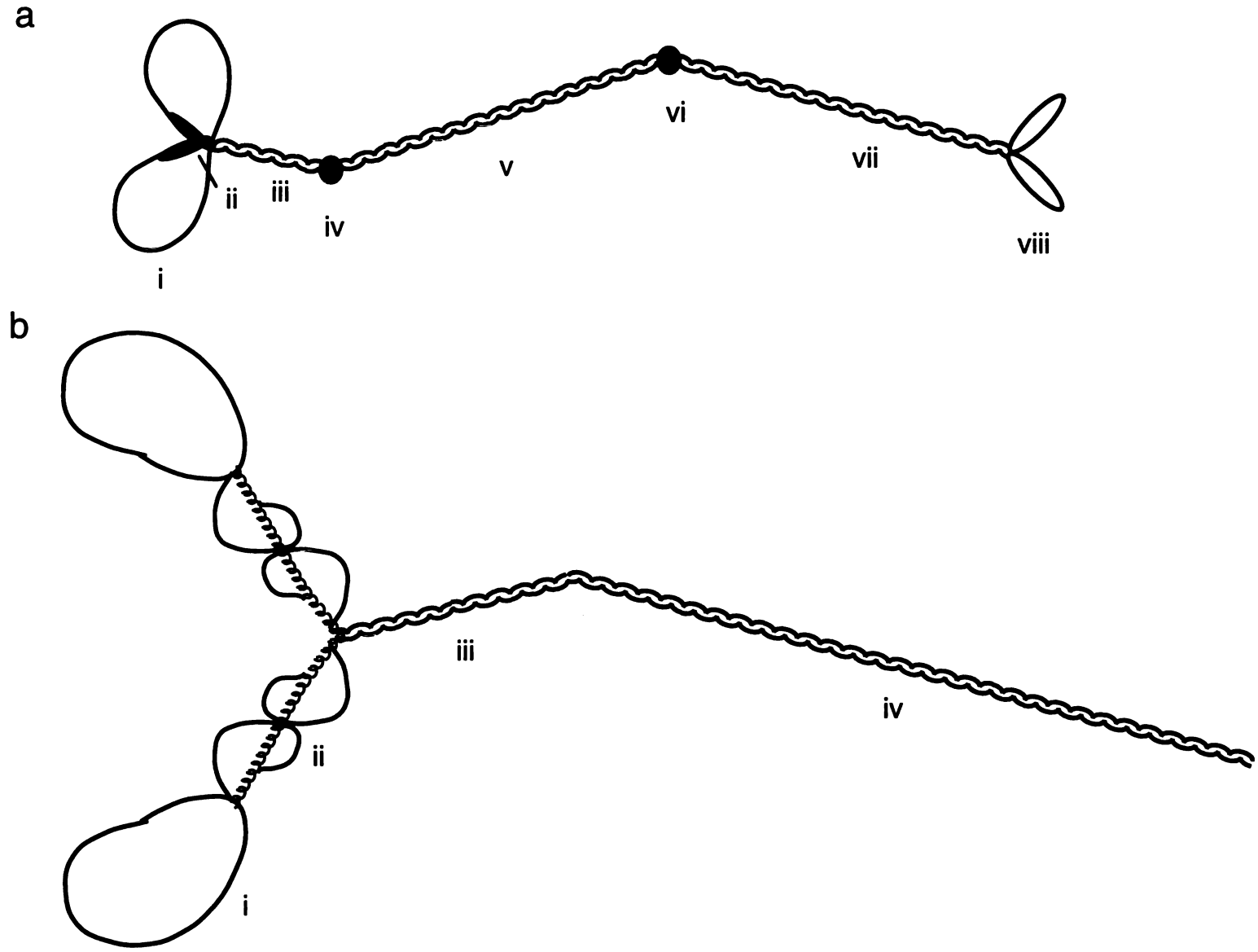
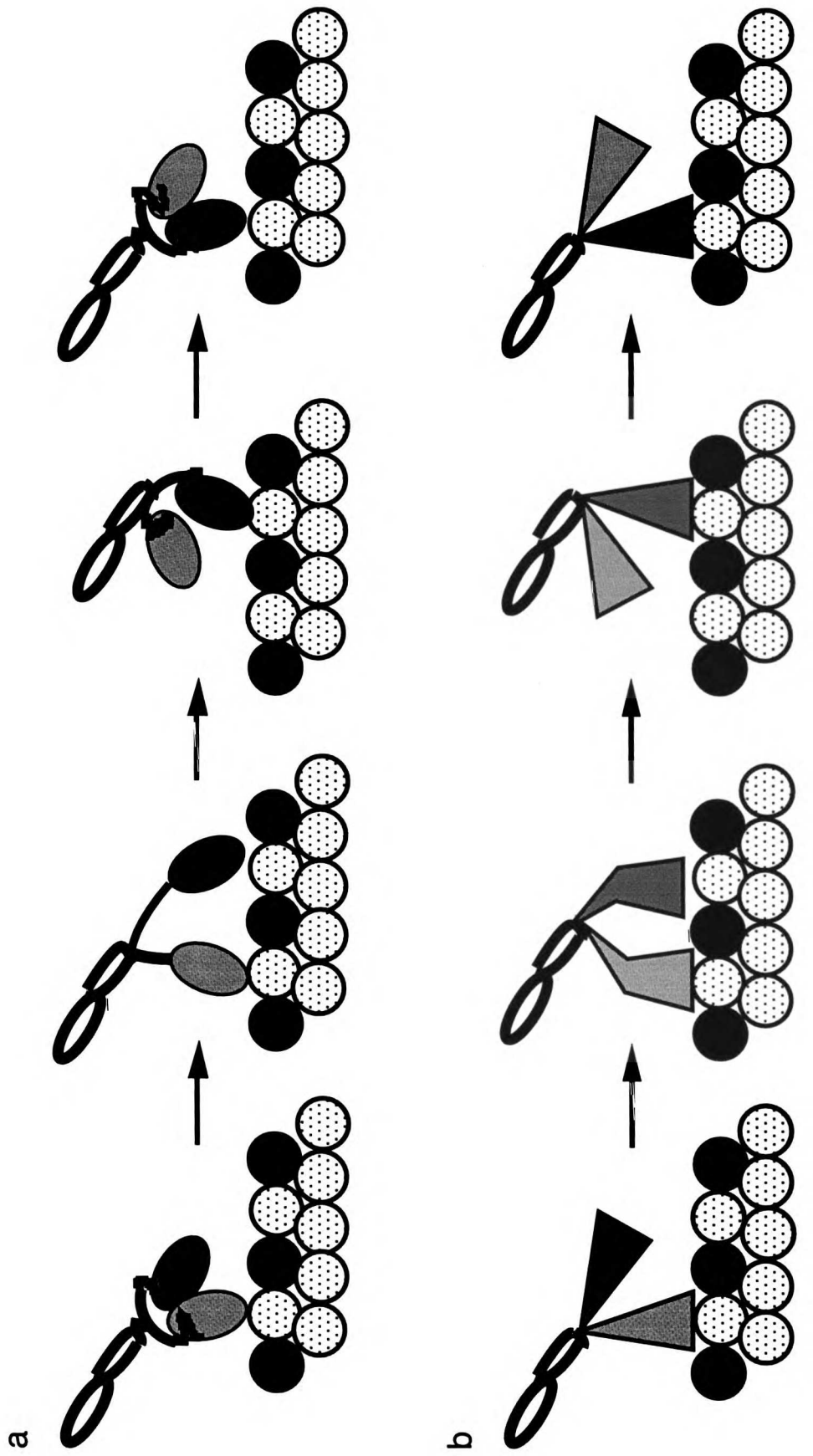

a
i
ii
iii
iv
v
vi
vii
viii
b
i
ii
iii
iv

Figure 2: Models for movement of dimeric kinesin

In order for kinesin to bind to the microtubule with both heads simultaneously, the structure connecting the two heads must change. After the second head binds, reformation of the original structure pulls the first head off the microtubule. Two models for this process are possible: a) The connection between the heads extends and becomes more flexible, releasing the second head on a tether that allows it to undergo a diffusional search for the next tubulin binding site. b) The motor undergoes a conformational change, swinging the second head of the dimer closer to its binding site.


a
b

CHAPTER 1

Chemomechanical Cycle of Kinesin Differs from that of Myosin

Chemomechanical cycle of kinesin differs from that of myosin

Laura Romberg* & Ronald D. Vale†‡

Department of * Biochemistry and † Pharmacology, University of California, San Francisco, California 94143, USA

MOTOR proteins move unidirectionally along cytoskeletal polymers by coupling translocation to cycles of ATP hydrolysis. The energy from ATP is required both to generate force and to dissociate the motor–filament complex in order to begin a new chemomechanical cycle[1,2]. For myosin, force production is associated with phosphate release following ATP hydrolysis, whereas dissociation of actomyosin is tightly coupled to the binding of ATP[3]. Dynein, a microtubule motor, uses a similar cycle[4], suggesting that all cytoskeletal motors might operate by a common mechanism. Here we investigate kinesin's chemomechanical cycle by assaying microtubule movement by single kinesin molecules when intermediate states in the hydrolysis cycle are prolonged with ATP analogues or inhibitors. In contrast to myosin and dynein, kinesin with bound ADP dissociates from microtubules during translocation, whereas kinesin with unhydrolysed nucleotide remains tightly associated with the polymer. These findings imply that kinesin converts ATP energy into mechanical work by a pathway distinct from that of myosin or dynein.

Single kinesin molecules attached to a microscope slide can translocate microtubles for several microns at velocities identical to those produced by multiple motors[5,6]. As diffusion would separate a detached microtubule from the two-headed kinesin molecule within a millisecond, kinesin must remain bound to the microtubule for most of the ATPase cycle (~50 ms in total[7]). But each head must dissociate briefly when moving to a new tubulin binding site. To determine when this occurs in the hydrolysis cycle, ATP analogues and inhibitors were used to prolong different chemical intermediates (no nucleotide, ATP, ADP plus hydrolysed P_i (ADP·P_i) and ADP). Agents that prolong kinesin intermediates that are weakly bound should cause microtubules to dissociate more frequently from single kinesin molecules. Those that extend a strongly bound intermediate should allow single motors to move microtubules for many microns, albeit at slower rates. In addition, prolonging a strong binding state should increase the drag kinesin imposes on microtubules, thus causing transport by multiple motors to be slower than that produced by a single motor[8].

To assay single motor motility, kinesin was adsorbed onto a surface at a low density[5]. Microtubules that pivoted about single attachment points and travelled distances shorter than their

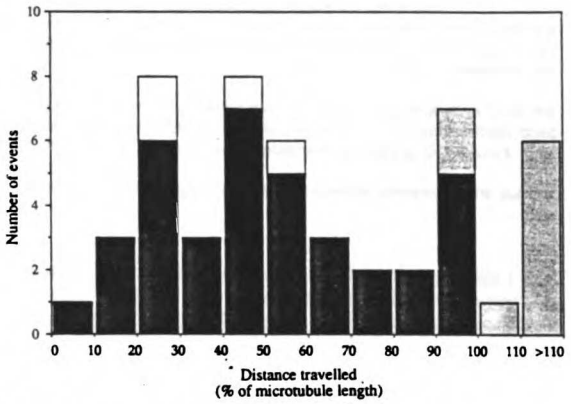


FIG. 1 Microtubule motility on surfaces sparsely coated with kinesin; criteria for establishing movement by single motors. This histogram shows how far microtubules travel before dissociating (expressed as a percentage of their length). Translocation events are subdivided according to whether microtubules moved in a straight line (open bars) or pivoted about one (solid bars) or two successive (shaded bars) points of attachment to the surface. Microtubules that moved further than their length always had a second pivot point, suggesting an interaction with a second kinesin motor. Only those microtubules with a single point of attachment and which did not move beyond their length were considered to have interacted with a single kinesin. Video observations indicate that the ends of microtubules in solution frequently contact the surface; thus initial attachment to motors may not occur at random along the length of a microtubule.

METHODS. Kinesin was purified from squid lobes by microtubule affinity[20] followed by sucrose gradient sedimentation (5–20% sucrose prepared in 80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA; spun at 200,000*g* for 12 h). Motility assays were performed as in ref. 5 with the following modifications. The surface of a microscope perfusion chamber was precoated for 2 min with casein (5 mg ml^{-1} in 10 mM Tris, pH 7.7, 150 mM NaCl) instead of cytochrome *c*/tubulin. A solution of 80 mM PIPES, pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$ was used for all subsequent dilutions and for assaying motility. Kinesin was introduced into the microscope chamber at a concentration of 4 μg ml^{-1} for the multiple motor assay, and 50 ng ml^{-1} for the single motor assay, yielding surface densities of 560 and 7 molecules per μm^2 respectively[5]. By applying Poisson statistics to the probability that a microtubule finds a second motor during translocation (moving 4 μm on average), we calculated that there was an average of one motor per 18 μm along the microtubule path in the single motor assay. Therefore in multiple motor assays about 20 motors were estimated to be attached to each 5 μm long microtubule. Before introduction into the microscope chamber, microtubules stabilized with 10 μM taxol (15 μg ml^{-1} in the multiple motor assay and 90 μg ml^{-1} in the single motor assay) were sheared with a 27-gauge needle (average length of 4–7 μm in different experiments). Microtubule motility was observed using dark-field optics. Velocities and distances translocated by single motors were determined by measuring the distance from the ends of microtubules (at the start and end of a translocation event) to the node about which they pivoted. All translocation events occurring within a 1,200 μm^2 field of view were measured.

‡ To whom correspondence should be addressed.

TABLE 1 Microtubule interaction times and velocities with different nucleotides

Nucleotide	Dissociation from single motors: Number of releases*	Seconds (μm) bound to MTs	Seconds bound per release	μm travelled per release	Velocity (μm s^{-1})†: One motor	Multiple motors	Ratio
1 mM ATP	17	186 (152)	11	9	0.82 ± 0.03	0.75 ± 0.01	1.1
2 μM ATP	29	4,297 (93)	148	3	0.022 ± 0.001	0.011 ± 0.001	2
250 μM ATP-γS‡	25	4,149 (67)	166	3	0.016 ± 0.001	0.012 ± 0.0003	1.4
1 mM ATP/150 μM ADP	39	212 (153)	5	4	0.72 ± 0.02	0.67 ± 0.01	1.1
1 mM ATP/250 μM ADP	—	0 (0)§	—	—	—	0.55 ± 0.01	—

Nucleotides were purchased from Boehringer Mannheim. ATP-γS was further purified by chromatography on a Mono-Q column (Pharmacia) with a linear 0–0.6 M triethylamine gradient. The final product was analysed by 0.3 M ammonium bicarbonate isocratic elution. It contained 0.5% ATP and 1% ADP; the latter increased to 5% over a period of one month. The ATP-γS, used shortly after preparation, was assayed with AP5A (P^1P^5-di(adenosine-5'-)pentaphosphate; 100 μM) to inhibit any dinucleotide kinase that may have been present. An ATP-regenerating system (0.1 mg ml^{-1} creatine kinase and 1 mM phosphocreatine) was included in the assay with 2 μM ATP.

* A translocating microtubule is considered to dissociate from a single motor if it is released into solution before its trailing end reaches the pivot point where the kinesin molecule is presumably located. For each condition, data were taken from multiple microtubules on at least three slides.

† The translocation velocities produced by one and multiple motors are expressed separately and as a ratio. Microtubules in multiple motor assays are estimated to be bound by ~20 kinesins (see Fig. 1). The means and standard errors from at least 60 velocity measurements are shown. It was difficult to determine whether ADP altered the velocity ratio because 150 μM ADP decreased microtubule velocity only slightly (~15%), whereas higher concentrations did not permit translocation by single motors.

‡ Two findings indicate that ATP-γS, and not a low level of contamination with ATP (0.5%), was responsible for the movement in these assays. First, the K_m for motility produced with ATP-γS was 8 μM (data not shown). At this concentration of total nucleotide, there is only 40 nM ATP, which by itself does not produce motility at detectable rates in these assays. Second, addition of 5 μM ATP (which on its own translocates microtubules at 0.025 μm s^{-1}) to a solution of 250 μM ATP-γS did not change the velocity significantly.

§ In the single motor assay, translocation events lasting less than 0.5 μm or 0.5 s could not be distinguished from nonspecific adhesion to the surface or collisions with the surface caused by diffusion.

length before dissociating were assumed to be transported by individual motors (Fig. 1). Occasionally a microtubule dissociated from the motor before its trailing end reached the pivot point. By dividing the number of these events by the total translocation time for all microtubules, an average interaction time was determined.

The time spent by kinesin in its nucleotide-free state was prolonged by decreasing the ATP concentration to 5-fold below the K_m for ATPase activity (10 μM; refs 7, 9). Under such conditions, microtubules moved by single kinesins dissociated 13-fold less frequently (once per 148 s; Table 1) than with 1 mM ATP (once per 11 s). The distance travelled per release was less than at high ATP concentrations, implying that dissociation occurs, albeit infrequently, during the nucleotide-free state. As seen previously[5], single motors translocated microtubules at twice the velocity of multiple motors in a high-density assay, indicating that nucleotide-free kinesin binds strongly to microtubules and imposes a drag on movement.

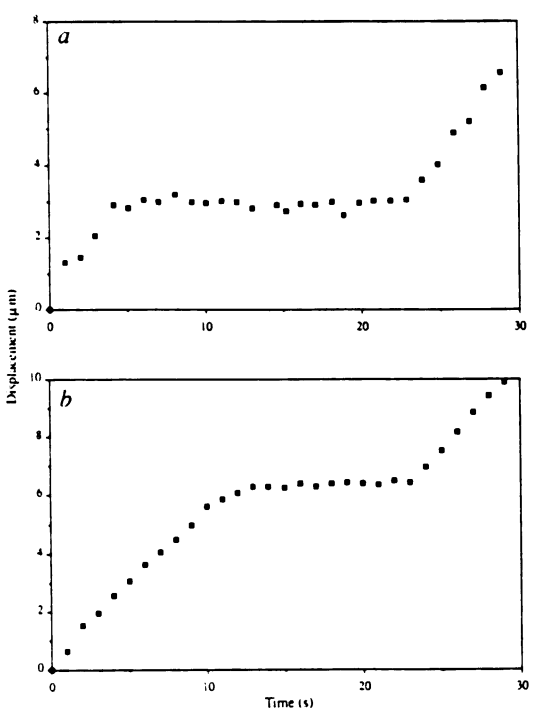


FIG. 2 Vanadate (50 μM) causes intermittent disruptions in microtubule transport in the presence of 1 mM ATP. The displacement of representative microtubules as a function of time at single (*a*) and multiple (*b*) motor densities is shown. The average velocities are 0.63 and 0.58 μm s^{-1} during translocation for *a* and *b* respectively. Paused microtubules resume translocation less frequently when either the motor density or the vanadate concentration is raised. But restarting occurred in almost all cases in the single motor assay with 50 μM vanadate. Microtubule ends (±0.2 μm error) were tracked using a custom computer program.

ATP-γS is an analogue whose rate of hydrolysis, and possibly product release, is slow compared with that of ATP[10,11]. In the presence of 250 μM ATP-γS, single motors translocated microtubules slowly (0.012 μm s^{-1}) and remained bound for a time (166 s) comparable to that observed with 2 μM ATP. The velocity ratio (single/multiple motors) of 1.4, which is between that of 2 μM ATP and 1 mM ATP ($P < 0.001$), suggests that ATP-γS prolongs a tightly bound microtubule state.

Two phosphate analogues, aluminium fluoride (250 μM) and vanadate (50 μM), were added to 1 mM ATP to prolong an intermediate similar to kinesin-ADP·P_i. In single and multiple motor assays with these analogues, some microtubules stopped translocating but remained bound, while others continued to move at close to full speed. Similar results have been found previously with vanadate in high-density assays[12]. Immobile microtubules in the presence of aluminium fluoride rarely resumed movement. In the presence of vanadate, however, paused microtubules often resumed translocation (Fig. 2), perhaps because of an occasional dissociation of the inhibitor. Thus, both phosphate analogues cause strong binding of kinesin to microtubules. In contrast to the findings with 2 μM ATP and 250 μM ATP-γS, this strong binding state can resist the actions of other motors generating force on the same microtubule.

To extend a kinesin-ADP state, Mg-ADP was added as a competitive inhibitor to 1 mM ATP. The addition of 150 μM

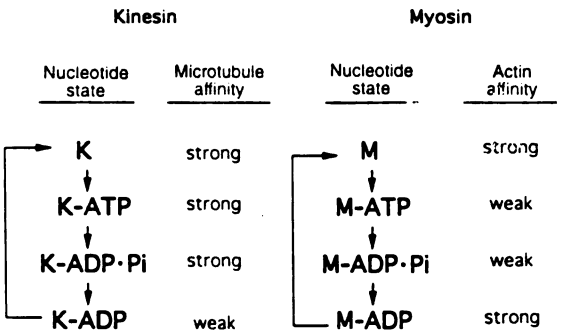


FIG. 3 Models for the filament binding interactions of kinesin (K) and myosin (M) during the ATP hydrolysis cycle. Possibilities of multiple nucleotide states or mechanical influence are not included in this scheme.

ADP caused microtubules to dissociate from single kinesins twice as frequently; no binding was detected with 250 μM ADP (Table 1). Similar concentrations of ADP in the absence of ATP also dissociated microtubules from kinesin (data not shown). These results suggest that ADP weakens the binding between kinesin and microtubules. The sudden suppression of translocation with increasing ADP may reflect the need for both kinesin heads to detach simultaneously in order for a microtubule to dissociate. Consistent with this idea, higher concentrations of ADP (5–20 mM) were required to dissociate microtubules in the multiple motor assay.

These results suggest the following sequence of interactions between kinesin and microtubules during the ATP hydrolysis cycle (Fig. 3). In the nucleotide-free state, kinesin is strongly bound to microtubules. After nucleotide binding, the motor remains tightly associated with the polymer, yet some conformational change must occur that diminishes its ability to resist microtubule translocation in a multiple motor assay. Kinesin-ADP·Pi is still strongly bound to the microtubule, but after phosphate release, the interaction becomes weak, potentially allowing the motor to dissociate and find a new binding site. This model of the kinesin cycle requires the assumption that the analogues and inhibitors used here prolong the predicted states and that these states mimic normal intermediates in hydrolysis. Transient-state kinetic measurements[13,14] of kinesin dissociation during the normal ATPase cycle will be needed to confirm this model.

In a motor protein's chemomechanical cycle, energy is required to dissociate the strongly bound motor–polymer complex and to produce force. Our results suggest that the utilization of ATP energy by kinesin is different from that of other cytoskeletal motors. Upon binding ATP, myosin[13] and dynein[14,15] rapidly dissociate from their filaments. Although the binding of ATP to kinesin is associated with a free energy change[16] of similar magnitude to that of other motors[17,18], our results indicate that kinesin does not dissociate from microtubules early in its hydrolysis cycle. How then does kinesin use the binding energy of ATP? One possibility is that the energy may be stored in the protein for use later in the cycle, as occurs for other proteins such as bacteriorhodopsin[19]. Alternatively, the binding of ATP may be directly coupled to mechanical work. This issue can be resolved by determining when force production occurs in the hydrolysis cycle. □

Received 28 September; accepted 2 November 1992.

1. Eisenberg, E. & Hill, T. L. *Science* **227**, 999–1006 (1985).
2. Jencks, W. P. in *The Roots of Modern Biochemistry* 571–580 (Walter de Gruyer, Berlin, 1988).
3. Cooke, R. *CRC crit. Rev. Biochem.* **21**, 53–118 (1986).
4. Johnson, K. A. *A. Rev. Biophys. biophys. Chem.* **14**, 161–188 (1985).
5. Howard, J., Hudspeth, A. J. & Vale, R. D. *Nature* **342**, 154–158 (1989).
6. Block, S. M., Goldstein, L. S. & Schnapp, B. J. *Nature* **348**, 348–352 (1990).
7. Hackney, D. D. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6314–6318 (1988).
8. Pate, E. & Cooke, R. *J. Musc. Res. Cell Motil.* **12**, 376–393 (1991).
9. Kuznetsov, S. A. & Gelfand, V. I. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8350–8354 (1986).
10. Goody, R. S. & Mannherz, H. G. in *Protein–Ligand Interactions* (eds Sund, H. & Blauer, G.) (Walter de Hruyer, Berlin, 1975).
11. Shimizu, T., Katsura, T., Domanico, P. L., Marchese-Ragona, S. P. & Johnson, K. A. *Biochemistry* **28**, 7022–7027 (1989).
12. Cohn, S. A., Ingold, A. L. & Scholey, J. M. *J. biol. Chem.* **264**, 4290–4297 (1989).
13. Lymn, R. W. & Taylor, E. W. *Biochemistry* **10**, 4617–4624 (1971).
14. Porter, M. E. & Johnson, K. A. *J. biol. Chem.* **258**, 6582–6587 (1983).
15. Omoto, C. K. & Johnson, K. A. *Biochemistry* **25**, 419–427 (1986).
16. Sadhu, A. & Taylor, E. W. *J. biol. Chem.* **267**, 11352–11359 (1992).
17. Taylor, E. W. in *Heart and Cardiovascular System* (eds Fozzard, H. A.) 1281–1293 (Raven, New York, 1992).
18. Holzbaur, E. L. F. & Johnson, J. A. *Biochemistry* **28**, 428–434 (1986).
19. Mathies, R. A., Lin, S. W., Ames, J. B. & Pollard, W. T. *A. Rev. Biophys. Chem.* **20**, 491–518 (1991).
20. Vale, R. D., Reese, T. S. & Sheetz, M. P. *Cell* **42**, 39–50 (1985).

ACKNOWLEDGEMENTS. We thank F. Malik for purifying kinesin, T. Shimizu and T. Mitchison for advice on purification of ATP-γS, W. Marshall for developing the measurement program, R. Cooke for discussion, and F. Malik, G. McCaffrey and F. McNally for critically reading the manuscript. This work was supported by a grant from the NIH.

CHAPTER 2

Heterodimeric, Single-Headed Kinesin

Single molecules of kinesin are processive, moving along microtubules for microns without releasing. Models for processivity have been proposed in which the two heads of the dimer coordinate to move hand-over-hand along the microtubule (Block, et al., 1990; Gilbert, et al., 1995; Hackney, 1994; Howard, et al., 1989; Schnapp, et al., 1990). However, other mechanisms could produce single motor motility. If the motor released only briefly (<1 ms) while stepping to a new tubulin binding site (Block, et al., 1990; Howard, 1996; Leibler and Huse, 1993; Spudich, 1990), or if more than one binding site existed per tubulin dimer (Tucker and Goldstein, 1997), kinesin might be able to move along the microtubule without diffusing away. Processivity would then be enhanced by dimerization, but would be based on the properties of an individual kinesin head.

In order to distinguish a processivity mechanism based on the characteristics of the individual motor domain from one in which the two heads need to cooperate with each other, I decided to examine whether a monomeric motor domain could move as a single molecule. However, forming a monomer by simply deleting kinesin's dimerization regions can alter the protein's ATP hydrolysis and motility properties (Huang and Hackney, 1993; Jiang, et al., 1997; Stewart, et al., 1993). In addition, the presence of the dimeric stalk is necessary to adhere the motor to the microscope slide surface during microtubule gliding assays (which were the only single motor assay available in the lab at the time). Furthermore, it was unknown whether sequences C-terminal to the heads contributed structural characteristics to the motor that were important for single molecule motility. To eliminate these concerns, I decided to generate a kinesin molecule that possessed a dimeric stalk domain with only a single motor head.

A molecular biological approach to creating such a protein was adopted. Two polypeptides were simultaneously co-expressed, one containing a complete motor domain and one that had been truncated to eliminate the N-terminal motor head (Figure 1). The C-terminus of both polypeptides included ~130 a.a. of coiled-coil stalk sequence (up to a.a. 560), allowing the proteins to dimerize. In preliminary constructs, residue 385 was chosen for the start site of the truncated protein because conservation in the motor domain of conventional kinesin extends until this point. Co-expression of the two polypeptides was expected to result in a mixture of single-headed heterodimers with homodimers possessing either two or zero heads. As controls, wild type proteins possessing two heads (a.a. 1-560), or stalk dimers with no heads (a.a. 385-560), were expressed separately.

The two polypeptides needed to be expressed in equivalent amounts in order to form the highest proportion of heterodimer. To achieve this, two open reading frames (ORFs) were introduced into a single bicistronic RNA (Figure 1). The inclusion of a Shine-Dalgarno sequence between the ORFs allowed the ribosome to remain bound to the RNA between translation of the two proteins (Guzman, et al., 1992; Schoner, et al., 1986). However, this initial bicistronic plasmid was found to express polypeptide from the first ORF at much higher levels than that from the second. Two factors might have contributed to this. Both the number of bases between the Shine-Dalgarno sequence and the 5' end of the second ORF, and the secondary structure of this 5' region can alter the expression level of the second polypeptide. Altering the number of bases after the Shine-Dalgarno sequence did not change the expression levels of the polypeptides. However, when the start site for the second, headless polypeptide was moved (from a.a. 385 to a.a. 350), the new plasmid was found to express from both ORFs equally. In

addition, this new construct included a greater portion of the kinesin neck, which was a region that had recently been shown to contribute to dimerization (Correia, et al., 1995; Huang, et al., 1994). Corresponding plasmids for the control stalk homodimer (a.a. 350-560) were also recloned.

In order to purify the desired single-headed heterodimer away from wild type homodimers with two heads, an epitope tag was fused to the C-terminus of the headless polypeptide. Two tags (Glu-Glu: EEYMPME, Bourne lab; and strep tag: SAWRHPQFGG, Biometra) were tested before one was found (Flag tag, Kodak) that was detectable by western blot when fused to the C-terminus of the protein. The motors were then purified either on a Flag antibody column followed by a mono-Q column (for the bicistronic or stalk construct), or on a phosphocellulose column followed by a mono-Q column (for k560). Both the k560 and the stalk homodimers bound to mono-Q resin and eluted at ~0.29 and 0.25 salt respectively, so this column provided purification away from bacterial proteins, but only minimal separation of different kinesin products.

A preliminary protein preparation from the bicistronic construct was found to contain polypeptides corresponding to both ORFs. In the desired heterodimer, the untagged polypeptide would be expected to bind to the Flag column because it is dimerized with the tagged polypeptide. To test this protein for processivity, the kinesin was diluted to various concentrations and observed in microtubule gliding assays. The gliding velocity, dilutability, and MT pivoting behavior observed were all indistinguishable from those of wild type k560.

Single molecule assays are extremely sensitive to contamination with small amounts of wild type protein. It was therefore important to determine whether the untagged two-headed motor was being efficiently purified away

by the Flag column. A control, untagged k560 construct was incubated with Flag antibody resin, and surprisingly, the protein was found both to bind to the resin and to elute when the Flag peptide was applied. This result might be explained by the high charge of the Flag epitope, since kinesin is known to adhere to ionic surfaces. Washing the column with 0.5 M NaCl substantially reduced nonspecific binding, but untagged kinesin still bound and eluted with yields that were as much as 10% those of the tagged protein. Serendipitously, it was discovered that histidine-tagged k560 did not detectably bind to and elute from Flag columns. Therefore, a second bicistronic construct was made in which a histidine tag was fused to the C-terminus of the full length k560 polypeptide.

The his-tagged bicistronic protein was purified over Ni^{2+} and mono-Q columns and again tested for motility and processivity. During gliding assays on surfaces densely coated with this protein, many microtubules moved slowly or not at all, while a few moved at wild type speeds. As the motor was diluted, moving microtubules often wobbled or diffused away from the surface. At the lowest kinesin dilutions, most of the microtubules at the surface moved rapidly and pivoted around their attachment points, indicating processive motility. The above results are suggestive of a mixed population of motor in which slower-moving single headed proteins do not bind well to microtubules, but contaminating two-headed motor moves the polymer rapidly and processively.

In order to determine whether the histidine-tagged heterodimer was still contaminated with wild type k560, it was necessary to analyze the protein for the presence of zero-, one- or two-headed species, despite the similar chemical and structural properties of these proteins. Results from three

techniques which separate proteins on the basis of structure are described below.

When control homodimeric proteins (k560 and stalk) were spun over 5-20% sucrose gradients, the two proteins separated into neighboring fractions, with the full length band running faster than BSA and the stalk running more slowly. A single-headed heterodimer would be expected to show an intermediate mobility, with both polypeptides co-migrating on the gradient. Instead, when the bicistronic preparation was examined, the two bands overlapped but did not coincide. The full length band again appeared in the fractions just below BSA, while the stalk band now co-migrated with BSA. Further experiments ruled out chain exchange during the course of the experiments. Therefore, the initial bicistronic preparation must have contained a mixed population of proteins. Although the results could not provide a definitive description of the protein population, it was clear that a homogenous population of one-headed kinesin had not been obtained.

Velocity analytical ultracentrifugation also indicated the presence of a mixed protein population (Figure 2). Experiments with controls gave discrete S values of 2.5 ± 0.3 and 4.2 ± 0.3 (mean ± standard deviation of 2-3 runs) for the stalk and k560 homodimers respectively. However, the bicistronic protein seemed to be a mixture of proteins with S-values between 2.3-3.8 (reproduced in 2 runs). Although these experiments again did not define the exact composition of the mixture, they ruled out the possibility of a uniform population with an S-value appropriate for a one-headed kinesin.

Finally, rotary shadowing electron microscopy images of the protein were obtained by Ania Kashina at U.C. Davis. These images showed that at least two types of proteins were present in the preparations, confirming the results from the above two techniques. One protein was elongated with a

small globular domain at the end, while the other consisted of just the elongated portion of the protein. It was unclear whether a single head would be large enough to produce a visible globular domain, and so an identity could not be assigned to the specific proteins visualized. However, these results demonstrated that more than one species was present in significant quantities in the bicistronic preparation.

Taken together, the above experiments indicated that I had not achieved the goal of purifying a single headed kinesin, and that further attempts to do so might not be trivial. Therefore, the project was discontinued in favor of one studying processivity in ways that would not require the separation of kinesin species, and that would in the end be able to yield more detailed information about the relation between kinesin structure and processivity.

Figure 1: The Bicistronic Construct

The coding region for two polypeptides, one containing the kinesin motor domain plus half of the stalk (a.a. 1-560), and one containing just the stalk (either a.a. 385-560 or a.a. 350-560) were cloned into a single bicistronic RNA. Between the open reading frames is a Shine-Dalgarno sequence. In this version of the construct, there is a 3 base pair linker before the second open reading frame, and there is a histidine tag (HHHHHH) and a Flag tag (EEEEYMPME) at the 3' end of the first and second open reading frames respectively. Other versions contained 6 or 9 base pair linkers, or omitted the histidine tag.

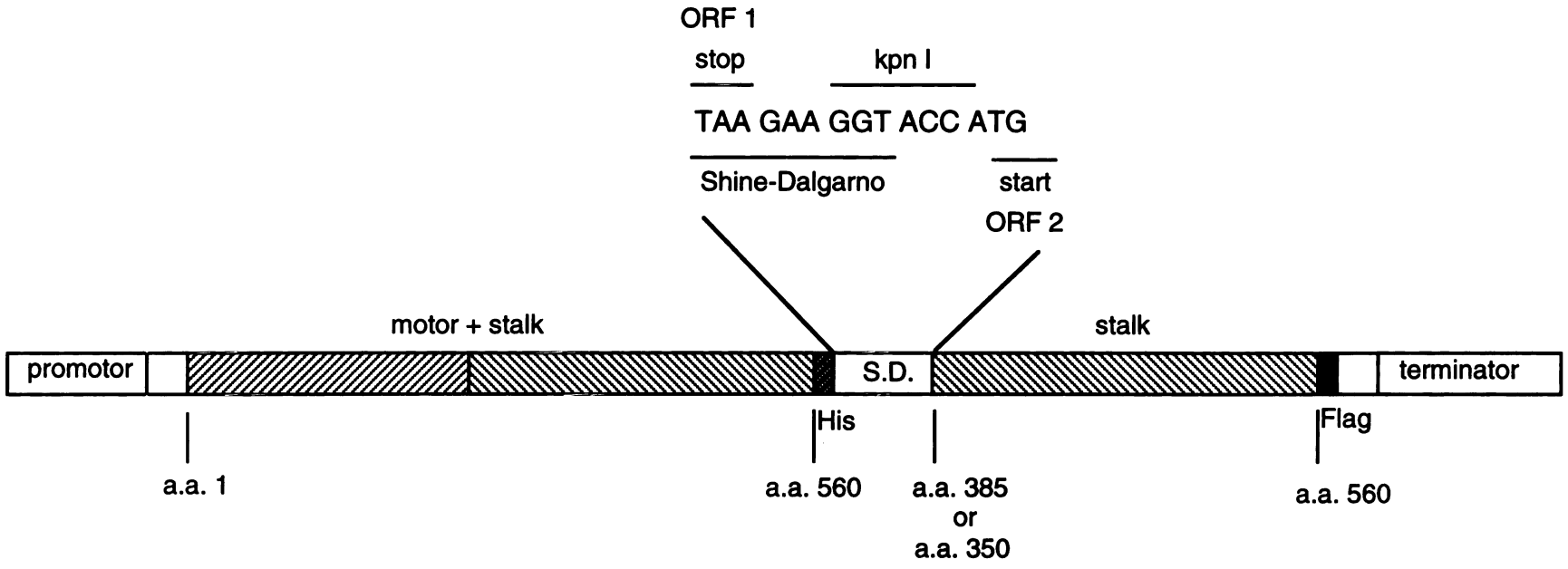
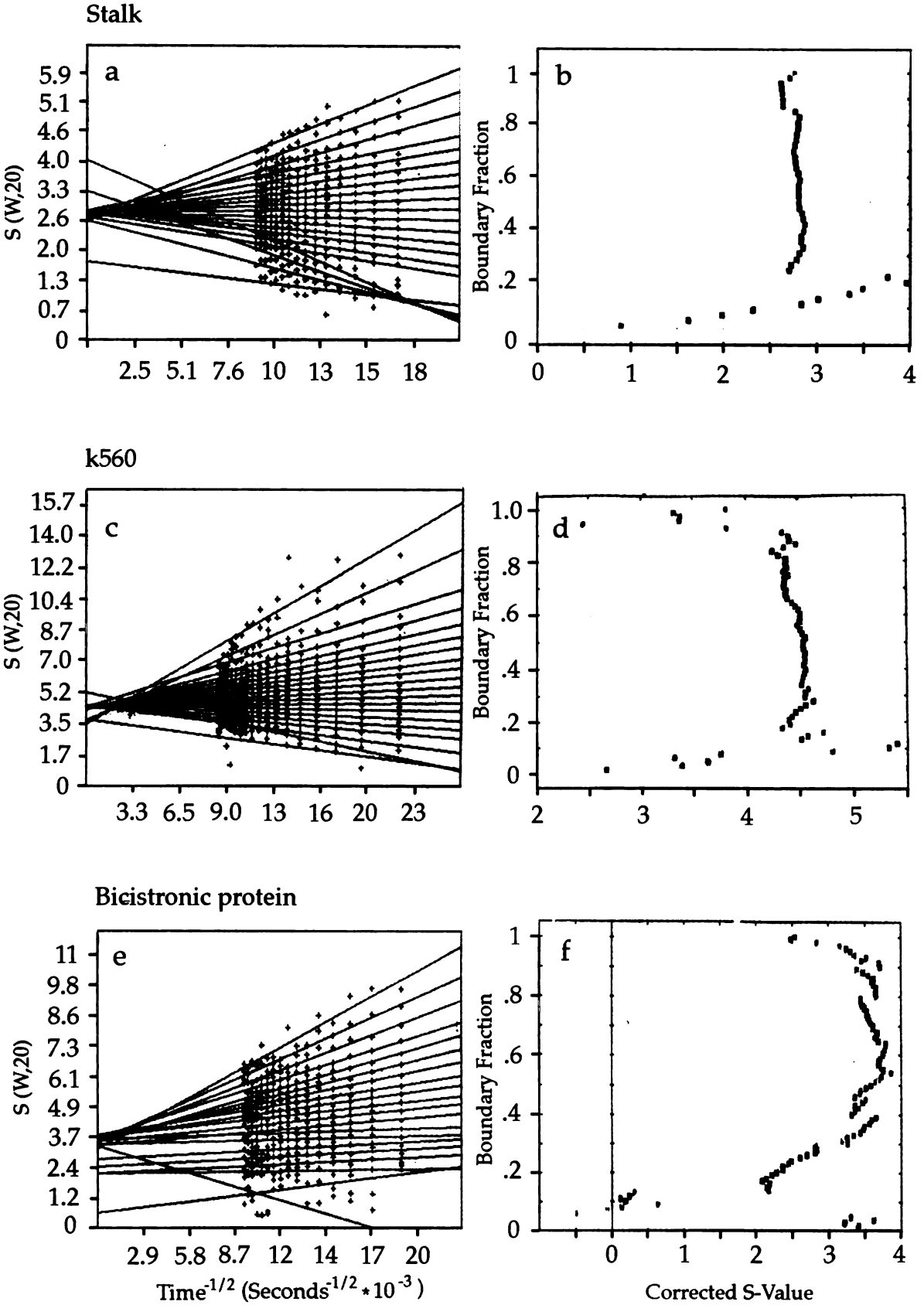

ORF 1
stop
kpn I
TAA GAA GGT ACC ATG
Shine-Dalgarno
start
ORF 2
motor + stalk
stalk
promotor
S.D.
terminator
His
Flag
a.a. 1
a.a. 560
a.a. 385
or
a.a. 350
a.a. 560

Figure 2: Hydrodynamic analysis of kinesin with zero, one, or two heads

S-values were determined for kinesin using a van Holde-Weischet velocity sedimentation analysis (van Holde and Weischet, 1978). a-b) Zero-headed stalk homodimers (a.a. 350-560 plus a Flag tag) sedimented with an S-value of 2.5. c-d) Two-headed K560 homodimers (a.a. 1-560) sedimented with an S-value of 4.2. e-f) Kinesin expressed from the bicistronic construct did not sediment with a uniform S-value (ranging from 2.3-3.8 S), indicating that it was not a homogenous population of single-headed heterodimers. *Methods*: Kinesin (~0.2-0.5 mg/ml in 25 mM Pipes 6.8, 0.2 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$) was spun for 3 hours at 35,000 RPM in a Beckman Optima XL-A Analytical Ultracentrifuge. Scans of the OD_{223} were taken across the length of the cell at 10 minute intervals. The van Holde-Weischet points were plotted (a, c, and e), indicating the apparent S-value of ten fractions of the sedimenting boundary at each time point during the spin. The calculated S-value was determined by extrapolating the data from each boundary fraction to infinite time (b, d, and f).


Stalk
a
b
S (W,20)
Boundary Fraction
5.9
5.1
4.6
4.0
3.3
2.6
2.0
1.3
0.7
0
2.5 5.1 7.6 10 13 15 18
1
.8
.6
.4
.2
0
0 1 2 3 4
k560
c
d
15.7
14.0
12.2
10.4
8.7
7.0
5.2
3.5
1.7
0
3.3 6.5 9.0 13 16 20 23
1.0
2 3 4 5
Bicistronic protein
e
f
11
9.8
8.6
7.3
6.1
4.9
3.7
2.4
1.2
0
2.9 5.8 8.7 12 14 17 20
Time-1/2 (Seconds-1/2 * 10-3)
0 1 2 3 4
Corrected S-Value

CHAPTER 3

Role of the Kinesin Neck Region in Processive Microtubule-Based Motility

Abstract

Kinesin is a dimeric motor protein that can move along a microtubule for several microns without releasing (termed processive movement). The two motor domains of the dimer are thought move in a coordinated, hand-over-hand manner. A region adjacent to kinesin's motor catalytic domain (the neck) contains a coiled-coil that is sufficient for motor dimerization and has been proposed to play an essential role in processive movement. Recent models have suggested that the neck enables head-to-head communication by creating a stiff connection between the two motor domains, but also may partially unwind during the mechanochemical cycle to allow movement to new tubulin binding sites. To test these ideas, we mutated the neck coiled-coil in a 560 a.a. dimeric kinesin construct fused to green fluorescence protein (GFP), and assayed processivity using a fluorescence microscope that can visualize single kinesin-GFP molecules moving along a microtubule. Our results show that replacing the kinesin neck coiled-coil with a 28 residue peptide sequence that forms a highly stable coiled-coil does not greatly reduce the processivity of the motor. This result argues against models in which unwinding of the coiled-coil is essential for movement. Furthermore, we show that deleting the neck coiled-coil decreases processivity by 10-fold, but surprisingly does not abolish it. We also demonstrate that processivity is increased 3-fold when the neck helix is elongated by seven residues. These results indicate that structural features of the neck coiled-coil, while not essential for processivity, can tune the efficiency of single molecule motility.

Introduction

Conventional kinesin is a motor protein that transports membranous organelles along microtubules *in vivo* (Bloom and Endow, 1995). Motility assays *in vitro* have shown that kinesin is capable of moving across hundreds of tubulin dimers (>1 μm) without detaching and diffusing away from the microtubule (Block, et al., 1990; Howard, et al., 1989; Vale, et al., 1996). Such processive movement is an unusual feature for cytoskeletal motor proteins (Howard, 1997) and does not appear to occur in muscle myosin (Finer, et al., 1994), ciliary dynein (Vale, 1992), or even other members of the kinesin superfamily (Case, et al., 1997). *In vivo*, processivity may be an important adaptation that allows efficient transport of organelles with only a small number of kinesin motors.

The kinesin motor domain consists of a 320 amino acid core that contains the microtubule binding and ATP hydrolysis sites and whose three-dimensional structure has been solved by X-ray crystallography (Kull, et al., 1996; Sablin, et al., 1996). This core catalytic domain, also termed the motor "head", is shared by all members of the kinesin superfamily. Adjacent to this core catalytic domain is the "neck" region, which is defined by sequence conservation among specific classes of kinesin motors and which is thought to act in concert with the catalytic domain to produce movement (Vale and Fletterick, 1997). In conventional kinesin, the neck consists of two parts: 1) an N-terminal ~10 residue β-sheet motif that is shared with many other plus-end directed motors and may confer directional motion (Case, et al., 1997; Henningsen and Schliwa, 1997), and 2) a subsequent ~30 residue hydrophobic heptad repeat that gives rise to a coiled-coil structure (Huang, et al., 1994; Morii, et al., 1997; Tripet, et al., 1997). The coiled-coil portion of the neck is

highly conserved (~65% a.a. identity) among conventional kinesins, suggesting a specialized function (Huang, et al., 1994; Vale and Fletterick, 1997). A noteworthy conserved feature of this region that is that it is contains two unstable heptads in the middle of the coiled-coil (Huang, et al., 1994; Tripet, et al., 1997). The neck coiled-coil is terminated by a sequence containing glycine and proline residues. C-terminal to this potential hinge region, kinesin contains a long (~400 a.a.) coiled-coil stalk domain and a globular tail domain that may help to dock the motor onto organelles.

Kinetic studies have suggested a model for kinesin processivity in which the motor remains in continuous contact with the microtubule as the result of an alternating interaction of the heads with the polymer (Hackney, 1994; Ma and Taylor, 1997). This idea has been supported by cryo-electron microscopy images that show dimeric kinesin bound to the microtubule by one head, while the other is detached and oriented perpendicular to the microtubule axis (Arnal, et al., 1996; Hirose, et al., 1996). Thus, binding of one head to the microtubule may inhibit binding of the partner head, at least during some intermediates of the mechanochemical cycle. However, experiments with monomeric kinesin also suggest that one head may help to release the other from its tubulin binding site at another stage of the cycle (Jiang and Hackney, 1997). By coordinating the two heads such that the binding of one head induces the release of the partner head, the motor could move hand-over-hand from one tubulin dimer to the next.

Truncations of kinesin have shown that monomeric motors are not processive, although these motors are still competent to produce motility when many proteins interact simultaneously with the microtubule (Berliner, et al., 1995; Vale, et al., 1996). However, if the truncation is less severe and yields a dimeric protein, the motor remains processive. The protein sequence

that enables these latter constructs to dimerize resides within the kinesin neck (Huang, et al., 1994). Several recent models have proposed a mechanochemical cycle in which the neck also acts as a sophisticated communication link between the heads (Hackney, 1994; Hirose, et al., 1996; Tripet, et al., 1997). At the start of the proposed cycle, the neck joins the two heads tightly, so that only one head can interact strongly with the microtubule. A nucleotide-induced conformational change in the bound head is then transmitted to the neck, causing the coiled-coil partially to unwind. This unwinding releases the second head so that it can now reach to a new tubulin binding site through a diffusional search, creating an intermediate in which both heads are bound to the microtubule. A rezippering of the coiled-coil at the end of the cycle then pulls the first head off the microtubule. This regenerates the motor state present at the beginning of the cycle with one head bound and the other detached, except that the motor has moved forward by one tubulin dimer. In support of such a model, the crystal structure of the kinesin dimer reveals that the neck coiled-coil forms a tight connection between the two catalytic domains, suggesting that coiled-coil unwinding could be necessary for simultaneous binding of both heads to the microtubule (Kozielski et al., manuscript submitted). Moreover, studies of synthetic kinesin neck peptides reveal an unstable region in the middle of the coiled-coil segment that could facilitate partial melting of this structure (Tripet, et al., 1997).

In order to test the role of the kinesin neck coiled-coil in processivity, we have either deleted or made alterations in this region and then assayed the resultant proteins for processivity using a single molecule fluorescence motility assay. We show that stabilizing the neck coiled-coil only reduced processivity by <50%. Thus it is unlikely that the neck coiled-coil needs to

unwind substantially during motility. On the other hand, increasing the flexibility in the connection between the two heads by inserting a three residue glycine linker at the beginning of the neck reduced processivity by 60%. A more drastic mutation of deleting virtually the entire neck coiled-coil decreased processivity 10-fold but did not abolish it. Unexpectedly, duplicating the first heptad repeat of the coiled-coil enhanced processivity by 3-fold. Collectively, these results suggest that neck coiled-coil is not essential for processivity, but that features of this structure make single molecule motility more efficient.

Materials and Methods

Expression Constructs

A pET17b vector (Novagen, Inc.) containing the N-terminal 560 amino acids of human kinesin followed by a C-terminal histidine tag(Woehlke, et al., 1997) was used as the starting point for mutagenesis of the kinesin neck region. In order to construct the desired mutations in the neck region, different strategies combining PCR, QuikChange mutagenesis (Stratagene, Inc.), or annealing followed by DNA synthesis with Sequenase (Amersham, Inc.) were employed. The neck region was then sequenced to confirm that only the correct changes were introduced, and then a Nco I-Hind III fragment containing the altered neck segment was subcloned back into the wild type construct or into a 560 amino acid kinesin-GFP fusion (using the Ser65Thr variant of GFP, Case, et al., 1997; Pierce, et al., 1997).

Protein Expression and Purification

At least four protein preparations of each neck mutant were made: two of the mutant kinesin alone and two of the kinesin-GFP fusion. For each preparation, E. coli BL21 (DE3) was transformed with the expression plasmid construct, and a single colony was selected and grown in 0.5 ml LB/50 μM ampicillin for 8 hr at 37°C. 10 μl of this pre-culture was then inoculated into 2 l of TPM media (20 g/l tryptone, 15 g/l yeast extract, 8 g/l NaCl, 10 mM glucose, 2 g/l KH_2PO_4, and 50 μg/ml ampicillin) and grown for 15 hr at 25°C. The cells were then induced with 0.1 mM IPTG and grown for an additional 8 hr. Cells were spun at 2,000 x g for 10 min, and the pellets were frozen in liquid nitrogen and stored at -80°C.

Cells were resuspended in 40-80 ml buffer (50 mM $NaPO_4$ pH 8, 20 mM imidazole, 250 mM NaCl, 1 mM $MgCl_2$, 1 mg/ml Pefabloc (Boehringer

Mannheim), 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.1 μg/ml chymostatin) per liter of culture and disrupted in a French press at 0.8 MPa (18,000 psi). Insoluble material was removed by centrifugation at 11,000 x g for 30 min. One ml of Ni^{2+}-NTA resin (Qiagen, Inc.) was incubated with the supernatant on a roller at 4° C for 1 hr before the resin was transferred to a column. The column was washed 8 times with 12 ml of wash buffer (50 mM $NaPO_4$, pH 6, 60 mM imidazole, 250 mM NaCl, 1 mM $MgCl_2$, 25 μM ATP), or until the OD_{280} was below 0.05. 1 ml of elution buffer (wash buffer with 500 mM imidazole, pH 7) was then applied, and the resin was allowed to equilibrate for 10 min. The protein was eluted with an additional 4 ml of elution buffer and then diluted 5-fold with mono-Q column buffer (25 mM Pipes pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 25 μM ATP). The protein was then applied to a mono-Q column (Pharmacia, Inc.), which was washed with column buffer plus 100 mM NaCl or 200 mM NaCl for kinesin or kinesin-GFP proteins respectively. The column was eluted with a gradient up to 1 M NaCl , and the kinesin eluted at ~0.35 M NaCl. Peak fractions were stored in liquid nitrogen after the addition of 10% sucrose. To quantitate protein concentrations, samples were run at varying dilutions on SDS-PAGE gels with a standard curve of BSA and were then stained with Coomassie dye. The gels were then imaged with a CCD camera and optical densities were calculated using the program NIH Image. Protein concentrations were between 0.2-1.4 mg/ml depending on the kinesin construct, and the purity of the full length protein was approximately 75% and 50% for the kinesin and kinesin-GFP constructs respectively. Nearly all the contaminants were degradation products which contained the histidine-tagged C-terminus of the protein and which did not show detectable binding to microtubules.

ATPase Assays

Microtubule-stimulated ATPase rates were measured in a spectrophotometer using a coupled enzymatic assay (Catterall and Pederson, 1971) with details as described in Woehlke *et al.* (1997). In brief, microtubules were polymerized with 20 μM paclitaxel, 1 mM GTP, 4 mM $MgCl_2$, and 10% DMSO, centrifuged through a 50% glycerol cushion, and resuspended in low salt assay buffer (12 mM Na-Pipes pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA) with 20 μM paclitaxel. ATPase assays were performed in assay buffer with 12 mM NaCl, 1 mM ATP, and a coupled NADH oxidation system. The assays were performed with 65-250 nM kinesin and 10-15 different microtubule concentrations ranging from 0.02-30 μM, depending on the construct. The K_m(MT) and k_{cat} values were determined using a Kaleidagraph-based hyperbolic curve-fitting routine, and R values were between 0.97-0.99. At least two complete ATPase curves of varying microtubule concentrations were performed for each protein preparation.

Multiple Motor Motility Assays

Kinesin motility was assayed by using differential interference contrast microscopy to observe microtubules gliding across kinesin-coated coverslips. Kinesin (0.7-7 μM) was combined in BRB80 buffer (80 mM Pipes pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA) with 0.1 mg/ml casein, paclitaxel-stabilized bovine brain microtubules (~0.5 μM), an ATP regenerating system (40 μg/ml phosphokinase (Boehringer Mannheim), 150 μg/ml phosphoenol pyruvate, and 1 mM ATP), and an oxygen scavenging system (Harada et al., 1990)(0.2 mg/ml glucose oxidase, 36 μg/ml catalase, 22 mM glucose, and 0.5% β-mercaptoethanol). The mixture was then pipetted into a flow chamber consisting of a coverslip supported over a glass slide by two strips of double-stick tape. Microtubules at the coverslip surface were visualized using a Zeiss

Axioplan microscope equipped with a 63x, 1.4 N.A. objective and a Hamamatsu Newvicon camera. Over the course of 5 min, images of several fields were recorded onto sVHS tape. The speeds of >30 microtubules per protein preparation were measured using a custom written computer analysis program.

Single Motor Motility Assays

Individual kinesin-GFP fusion proteins were visualized moving along sea urchin sperm flagellar axonemes in a total internal reflection fluorescence microscope. The microscope (Pierce and Vale, in press) and assay (Pierce, et al., 1997; Vale, et al., 1996) are described in detail elsewhere. In brief, 1-8 nM kinesin-GFP was diluted into the low salt buffer described above for the ATPase assay, except that K-Pipes was used rather than Na-Pipes, since it increased the number of kinesin molecules moving along axonemes. Additional components included 7.5 mg/ml BSA (to minimize kinesin-GFP adsorption to the surface), the oxygen scavenging system described above, 1 mM ATP and Cy-5 labeled sea urchin axonemes (Gibbons and Fronk, 1979; Vale, et al., 1996). 5 µl of this solution was applied onto a fused silica slide, and then sealed under coverslip using rubber cement dissolved in heptane. Slides were illuminated with an argon laser (488 nm) at 10 mW, since this provided optimal single spot detection. Up to 3 fields were imaged for 5 min each, and data was recorded onto sVHS tape after 4-frame averaging with an Argus 20 image processor (Hamamatsu Photonic Systems).

Analysis of Processivity

For analysis of single motor motility, a motor concentration was used in which movements on the axonemes were frequent but not overlapping. In addition, background fluorescence from motors diffusing in solution had

to be sufficiently low so that fluorescent spots could be easily distinguished. Data was recorded from two different preparation of each construct.

For fluorescence intensity analyses, the intensity of all moving spots in a 1-3 μm area of an axoneme were measured; this was repeated until >50 spots had been measured for each construct. The fluorescence intensity of GFP diminishes slightly during illumination (Pierce, et al., 1997). To minimize this contribution, intensities of spots were measured <1 sec after contacting the axoneme. Data were taken from a single frame acquired using a four frame rolling average. To determine the spot intensity, a 15 x 15 pixel area around a spot was selected, and the total intensity was measured using an Argus 20 image processor (Hamamatsu Photonic Systems). The background intensity derived from an adjacent region was then subtracted from this value. As a control to determine the fluorescence intensity of the overall motor population, all non-moving spots that landed in an area on the slide adjacent to the axoneme during the same time period were analyzed in the same manner.

The observed run lengths and speeds of 150-250 moving fluorescent kinesin-GFP spots were measured using a custom computer analysis program. Only movements on long axonemes were measured (generally 8-18 μm, although for the less processive mutants, axonemes as short as 5 μm were sometimes analyzed) to minimize the chance that a kinesin-GFP would release from the microtubule simply by running off the end. To measure run lengths, small segments of the axonemes were first viewed to locate moving spots and then observed frame by frame in order to ensure that the exact starting and ending point of each run was determined. Only kinesin-GFP spots that were well separated from other fluorescent spots, could be clearly distinguished from the background, and moved smoothly and continuously

for at least 0.5 sec were chosen for analysis. By visually tracking the center of the diffraction limited spots, movements as short as 0.1 μm could be detected. However, since the error in measuring the length of these runs is high, only runs >0.2 μm were used in velocity calculations. The efficiency of detecting extremely short runs was low because the dwell time of the motor on the microtubule was very brief. In order to more efficiently locate short runs for the least processive proteins, GLY3 and DEL, the axonemes were divided into very short segments for visual inspection (~1 μm versus ~3 μm segments for the other constructs). In addition, because these two constructs moved more slowly than wild type, their dwell times on the microtubule were increased, raising the likelihood that very short movements could be detected. Therefore, for these mutants, a more complete set of data at the shorter run lengths could be gathered.

Histograms of observed run length were plotted for each construct and fit to an exponential curve using the program Origin (MicroCal Inc.). Although all runs which were detected were plotted, only data from runs longer than 0.25 μm were used for the curve fitting because of the inefficient detection of runs below this limit. Runs as short as 0.2 and 0.15 μm were used for fitting GLY3 and DEL respectively, because of the more efficient data collection described above. The average run length values thus generated are referred to as the observed run lengths. However the rate constant for photobleaching (k_{bleach}), as well as the motor release rate constant (k_{rel}), contributes to the observed rate of disappearance of kinesin-GFP spots (k_{obs}) according to the equation: $k_{obs} = k_{rel} + k_{bleach}$. k_{obs} is the reciprocal of the dwell time of the motor on the microtubule, where the dwell time = observed run length/ motor speed. k_{bleach} was determined by counting the disappearance of kinesin-GFP spots adsorbed to the surface at 2.5, 5, 10 and 20

mW. In each case, the disappearance showed an exponential decay with time (Vale, et al., 1996), and the rate constant varied linearly with the laser power (Pierce, et al., 1997). At the 10 mW laser power used in this study, the photobleaching rate was 0.10 sec^{-1} for a kinesin-GFP dimer (Pierce, et al., 1997; Pierce and Vale, in press). From k_{obs} and k_{bleach}, the actual run length of the motor in the absence of GFP photobleaching (k_{rel}) could be calculated from the above equations. This correction for photobleaching was confirmed experimentally by measuring run lengths of the DUP mutant at 4 and 10 mW laser illumination as described in the text.

Results

Design of Neck Coiled-Coil Mutants

To design mutations in the neck, we used information derived from circular dichroism studies of kinesin neck peptides as well as functional studies of kinesin proteins truncated within this region. Structural studies by Morii et al. (1997) and Tripet et al. (1997) revealed that peptides containing the heptad repeat sequence from the neck will form a coiled-coil and suggested that the C-terminal boundary of this coiled-coil lies at Gly371. The N-terminal boundary was less clearly defined, but was unlikely to begin before Cys330 (amino acid numbers quoted in this paper correspond to residues in the human kinesin sequence). Functional studies have shown that kinesin truncated at residue 332 displays a low microtubule gliding velocity (Stewart, et al., 1993; Vale, et al., 1996) and an abnormal basal ATP rate and K_m for microtubule stimulated ATPase activity (Huang and Hackney, 1993; Jiang, et al., 1997). Kinesin with ten additional residues displayed more normal motility and ATPase properties. Therefore, to insure robust motor activity, mutations were made after Trp340 for all constructs in this study. Very recently, the crystal structure of dimeric rat kinesin has been solved and reveals a coiled-coil between residues 337-370 (E. Mandelkow, personal communication). Thus, the region chosen for mutagenesis in this study encompasses virtually the entire neck coiled-coil. All mutations described below were made in the context of a human kinesin protein that remains dimeric irrespective of the neck structure due to the presence of the first 110 amino acids of the coiled-coil domain of the kinesin stalk (K560, Figure 1).

To test whether the neck coiled-coil is essential for processivity in the context of a dimer, the residues 341-370 were deleted from K560 (DEL, Figure

1). In this mutant protein, the initial β-strand region of the neck is connected to a non-conserved region preceding the stalk. This non-conserved region contains several glycines and prolines and may serve as a hinge that could account for the flexible behavior of kinesin observed in motility assays (Huang, et al., 1994; Hunt and Howard, 1993).

To perturb any tight connection between the two heads that could serve to communicate tension or positional information, a flexible linker of three glycines was inserted at residue 342 (GLY_3). Six amino acids from the heptad repeat remain N-terminal to the glycine linker, but this sequence alone is insufficient to form a stable coiled-coil (Su, et al., 1994; Tripet, et al., 1997). As a control for amino acid insertions at residue 342, the construct DUP was created in which the prior seven amino acids (T336-K342) were duplicated. This construct would be expected to extend the neck coiled-coil by one complete turn of the α-helix.

Finally, two constructs were made to test the model that the neck coiled-coil needs to unwind during the mechanochemical cycle. In the construct STABLE COIL, residues 343-370 were replaced by four copies of the heptad repeat sequence EIEALKA. Thermodynamic studies of this 28 a.a. peptide have shown that it forms an extremely stable coiled-coil with a ΔG for melting that is >20 kcal/mol (Su, et al., 1994; Tripet, et al., 1997). Since the energy derived from ATP hydrolysis is ~12 kcal/mol, it is unlikely that this sequence would readily unwind during motility. A second and less drastic construct (YEN→ILI) was made by changing three non-ideal amino acids at the hydrophobic interface of the coiled-coil to more stabilizing residues (Y344I, E347L, N351I). These three substitutions have been shown to stabilize dimers of a neck peptide (a.a. 344-383), increasing the melting energy from 8.5 to 11.2 kcal/mol (Tripet, et al., 1997).

ATPase and Multiple Motor Motility Assays

All mutant proteins were first assayed for ATPase and *in vitro* motility activities in order to determine whether mutations in the neck coiled-coil affect basic enzymatic and motor functions (Table 1). The microtubule-stimulated ATP turnover rates for four of the five of the neck constructs were similar to that of the wild type motor (k_{cat} of 15-19 ATP/sec/head for mutants versus 22 ATP/sec/head for wild type). On the other hand, STABLE COIL displayed a slightly higher k_{cat} (31 ATP/sec/head). In addition, most of the mutants exhibited an apparent K_m for microtubule stimulation of the ATPase activity (K_mMT) which was similar to that of the wild type construct. An exception, however, was DUP, whose K_mMT was significantly lower (0.3 μM tubulin for DUP vs. 0.96 μM tubulin for WT). All of the neck mutants were also able to move microtubules across a microscope slide surface under conditions where multiple motors were contacting each microtubule (Table 1). The speeds of movement ranged from 44-72% of wild-type K560. The above assays confirm that none of the neck coiled-coil mutations severely damaged motile or enzymatic activity.

Single Molecule Motility Assays

The behavior of single molecules of the neck coiled-coil mutants proteins was tested in a motility assay using a total internal reflection (TIR) microscope. This low background fluorescence microscope is capable of visualizing individual fluorescent molecules (Funatsu, et al., 1995) and has been used to observe individual kinesin proteins moving along axonemes (a 9 + 2 array of microtubules) from sea urchin sperm flagella (Vale, et al., 1996). As the fluorescent tag for assaying mutants in this study, we fused the

Ser65Thr mutant (Heim, et al., 1995) of green fluorescent protein (GFP) to the C-terminus of the mutant K560 constructs (Pierce, et al., 1997). The kinesin-GFP fusion proteins were first tested in the multiple motor microtubule gliding assay to ensure that the constructs remained active when fused to GFP. All mutant-GFP proteins moved microtubules, and they did so at speeds between 48-115% of wild type kinesin-GFP (Table 2).

To observe single motor motility, the kinesin-GFP proteins were diluted to nanomolar concentrations in a buffer containing 1 mM ATP and Cy5-labeled fluorescent axonemes. By TIR microscopy, fluorescent spots corresponding to individual wild type kinesin-GFP appear along the length of an axoneme and move continuously in a given direction before releasing (Pierce, et al., 1997). Kinesin-GFP in solution moves rapidly by Brownian motion and contributes to a faint blurred fluorescent background. Surprisingly, for all the mutant kinesin-GFP proteins, fluorescent spots were observed moving unidirectionally along axonemes. The velocities of the moving spots for the DUP, YEN→ILI, and STABLE COIL constructs were similar to wild type (Table 2). In contrast, DEL and GLY_3 moved at approximately half the speed of the wild type protein. These single motor speeds largely mirror those from multi-motor assays, and are probably the more reliable measure of relative motor velocity because only active motors contribute to the data collected.

It was important to establish that the single spot motility observed for kinesin mutants was produced by kinesin dimers and not by motor aggregates. Using similar assay conditions, previous experiments showed that fluorescent spots of K560-GFP were twice as bright as monomeric GFP, indicating that K560-GFP is dimeric under the conditions of the assay (Pierce, et al., 1997). Here, we measured the single spot fluorescence intensities of

neck coiled-coil mutants fused to GFP and found that they all have similar average intensities to K560-GFP (within 30%), indicating that these proteins are dimeric under our assay conditions as well.

It was still possible, however, that a small population of aggregated protein could account for the moving fluorescent spots observed in assays with the mutant proteins. To rule out this possibility, we compared the fluorescence intensities of moving spots to that of spots that were adhered to the slide surface. For all constructs, the intensity histograms for moving spots were similar to those for the glass adsorbed population (Figure 2). However, for several, most notably GLY3 and YEN→ILI, there is a shoulder of brighter spots in the moving kinesin population that is not present in the non-moving histograms. Nonetheless, even in the most extreme case of GLY3, it is apparent that the majority of the moving spots are of the correct intensity. Thus, protein multimers are likely to constitute only a small percentage of the moving spots observed in these assays.

To further confirm that a minor population of aggregated motors does not account for the motility seen, the activity level (movements/μm MT/min/nM kinesin) was determined for each protein preparation (Table 2). All mutant proteins displayed similar activity to the wild type protein, and one mutant, STABLE COIL, was even more active. These data further argue against a rare protein aggregate as the source of moving spots for the mutant proteins.

Single Molecule Run Lengths

To examine whether the neck coiled-coil mutations changed the extent of processivity, the distance that the motors moved from the time that a fluorescent spot appeared on the axoneme to the time when it disappeared

was measured for >150 moving spots. As found previously (Block, et al., 1990; Vale, et al., 1996), these distributions could be fit to an exponential curve (Figure 3), indicating that kinesin has a constant probability of releasing and diffusing away from the microtubule each time it takes a step. The observed disappearance of kinesin from axonemes, however, is the sum of two independent exponential processes: the release of kinesin from the axoneme and the photobleaching of the GFP (Block, et al., 1990; Vale, et al., 1996). The true rate of release of the motor can therefore be calculated from the equation: $k_{rel} = k_{obs} - k_{bleach}$. The photobleaching rate for GFP was previously determined to be 0.10 sec^{-1} at 10 mW (Pierce and Vale, in press; see also Material and Methods). The observed run lengths multiplied by k_{obs}/k_{rel} define the actual motor run lengths, which are shown in Table 2 and quoted throughout the text. For wild type kinesin, the corrected run length is 1.3 μm, similar to previous reported values (Block, et al., 1990; Vale, et al., 1996). Assuming a kinesin step size of 8 nm (the distance between tubulin dimers, Svoboda, et al., 1993), the wild type protein takes 166 steps on average before detaching and has a release probability per step (P_{off}/step) of 0.6%.

The two constructs that increase the stability of the neck coiled-coil, YEN→ILI and STABLE COIL, exhibited run lengths that were similar to those of wild type kinesin (TABLE 2). The run length of YEN→ILI was 1.1 μm, or 84% of the wild type distance. Even in the 28 a.a. replacement of the coiled-coil (STABLE COIL), the run lengths were 55% of the wild type protein. These results suggest that it is unlikely that the neck coiled-coil needs to unwind significantly in order for the motor to move processively.

Two constructs, DEL and GLY_3, had significantly shorter run lengths than wild type, moving only 0.14 and 0.50 μm respectively. These two GFP constructs were also the only ones that moved at significantly slower speeds

than wild type in the single motor assays (0.16 and 0.17 μm/sec vs. 0.31 μm/sec for wild type). These mutants nevertheless have a high probability of stepping from one tubulin subunit to the next without detaching. For DEL, $P_{off/step}$ is 5.6%, indicating that this motor has a ~95% chance of remaining attached after completing a step. Therefore, while the tight coupling of the two heads by the neck coiled-coil may make processivity more efficient, the coiled-coil is not essential for processive motion.

Interestingly, the construct DUP moved ~3.5 fold farther than wild type, a phenomenon shown by two independent preparations of the protein. The fluorescence intensity data in Figure 2 indicates that the lengthened runs are not simply due to a large population of multimeric protein. However, because DUP has the longest dwell time on the microtubule, the photobleaching rate contributes substantially to the determination of corrected run length (the corrected run length is 2.5-fold greater than the observed run length). To confirm that the calculated run length of this motor is correct, we measured DUP movements at two laser powers and found that the observed run lengths at 4 mW laser power were significantly greater than at 10 mW (3.0 μm versus 1.8 μm). The corrected run length remained approximately the same (4.9 μm versus 4.4 μm), confirming both the validity of the photobleaching correction procedure and the long run length of the DUP construct.

Discussion

Recent models for processivity have suggested that the kinesin neck coiled-coil coordinates the two heads of the motor as they alternate interacting with the microtubule (Hackney, 1994; Hirose, et al., 1996; Tripet, et al., 1997). These models are supported by experiments showing that kinesin dimers containing the coiled-coil are processive, while monomers that lack the coiled-coil are not (Berliner, et al., 1995; Hackney, 1995; Vale, et al., 1996). However, in these previous truncation studies, specific roles that the neck coiled-coil might have in processivity, beyond simply dimerizing two motor domains, could not be assessed. Here, we have altered the neck coiled-coil in the context of a stable kinesin dimer in order to evaluate how specific structural features of the neck contribute to motor processivity. The resultant proteins were assayed using a motility assay that involves direct visualization of single, fluorescently labeled kinesin molecules (Pierce, et al., 1997; Vale, et al., 1996). Surprisingly, we show that deletion of virtually the entire neck coiled-coil (a.a. 341-370) does not abolish processivity, although the resultant protein could not move as far the wild type protein (18 versus 166 steps). We therefore conclude that the neck coiled-coil is not essential to processivity, provided that the motor is dimerized by downstream sequences. However, we also demonstrate that mutations that alter structural features in the neck affect single motor run length. This provides information on the mechanism by which kinesin steps along the microtubule lattice, as discussed below.

The neck coiled-coil does not need to melt during processive motility

A notable feature of the kinesin neck is the placement of destabilizing residues with low hydrophobicity (Y344, E347, N351) at the interface of the

coiled-coil. The high conservation of this unstable region has led to the suggestion that the N-terminal three-fifths of the coiled-coil may unwind during motility (Huang, et al., 1994; Tripet, et al., 1997). According to such a model, stabilizing this region of the neck should decrease processivity and might even interfere with movement entirely. However, we find that the constructs YEN→ILI and STABLE COIL move 0.8 and 0.6 times as far as the wild type protein, demonstrating that increased stability in the neck coiled-coil does not prevent processive motility. The free energy of melting of the entire STABLE COIL sequence is >20 kcal/mol (Tripet, et al., 1997), which exceeds the energy from ATP hydrolysis. The energy barrier for a partial melting of STABLE COIL is expected to be significant as well, since the unfolding of coiled-coil structures is highly cooperative (Su, et al., 1994). In addition, because of the free energy differences between the altered neck sequences, unwinding would be expected to be slower in the mutant proteins compared to the wild type motor. However, single motor motility of YEN→ILI and STABLE COIL occurs at wild type speeds, suggesting that melting of the coiled-coil is not a rate-limiting step in cycle.

The results with STABLE COIL raise interesting questions as to how the motor steps along the microtubule. For the motor to be processive, a transient intermediate in which both heads are bound to the microtubule is expected to exist (Hackney, 1994; Hirose, et al., 1996; Tripet, et al., 1997). However, the crystal structure of the kinesin dimer reveals that the length of the linker sequence between the two heads is insufficient to allow both to bind simultaneously to adjacent tubulin dimers 8 nm apart on the protofilament (E. Mandelkow, personal communication). Thus, in order to extend the connection between the heads, some structure must melt. Since our results do not support unwinding of the neck coiled-coil, we favor a

model in which the bonds that hold the β-sheet region of the neck (residues 323-332) to the catalytic domain are broken during the hydrolysis cycle, enabling these residue to adopt an extended conformation. Structural modeling indicates that up to 10 nm of extra linker could be created by such an event, which would be sufficient to allow both heads to bind to the microtubule (not shown). Consistent with this hypothesis, these residues adopt a well-ordered β-sheet structure in one crystal (Kozielski et al., manuscript submitted), but are disordered in other crystal forms (Kull, et al., 1996).

Since Y344, E347, and N351 are not essential for processive movement, why are these residues so strikingly conserved? One possibility is that they play a role in the regulation of the kinesin motor. The tail of the full length kinesin protein has been shown to fold onto the head and inhibit activity (Hackney, et al., 1992), and sequences in the neck coiled-coil may be important for this interaction, (Hackney and Huang, 1993). It is possible that partial melting of the kinesin neck coiled-coil could serve some function in the regulation of motor activity by the tail domain.

The neck coiled-coil enhances processivity

Although the neck coiled-coil is not essential to single molecule motility, mutant proteins with the coiled-coil deleted or containing a three glycine insertion move only ~10% and ~40% as far as wild type respectively. In addition, these DEL and GLY3 constructs moved at half the wild type speed in single molecule assays. Thus, the native neck coiled-coil does make processive motility more efficient.

The presence of the coiled-coil in the neck might enhance single motor motility by at least three mechanisms. First, the coiled-coil could help to

align the two polypeptides of the dimer so that regions involved in positioning the two heads could more readily interact. The positioning of the heads relative to one another is thought to cause the motor domains to undergo alternate cycles of microtubule-catalyzed ATP hydrolysis (Amos and Hirose, 1997; Hackney, 1994; Hirose, et al., 1996). Second, processivity may require tension to be transmitted through a stiff connection between the motor domains in order to release the posterior head from the microtubule (Jiang and Hackney, 1997). The slower single motor speeds of the DEL and GLY3 mutants might be explained by inefficient release of the posterior head from the microtubule, resulting in futile cycles of ATP hydrolysis without forward progress along the microtubule. Finally, charged residues on the outer surface of the coiled-coil could potentially interact with tubulin, which would help to maintain contact between the motor and the microtubule and thereby help processivity.

Surprisingly, we also discovered that duplicating the first heptad repeat of the neck (T336-K342) results in a striking "gain-of-function" phenotype, increasing processivity >3-fold. Thus, the length of the neck helix may play a role in the efficiency of processive movement. Interestingly, the neck helix of conventional kinesin motors is 10 residues longer than the predicted neck helices of other N-terminal motors in the kinesin superfamily (Vale and Fletterick, 1997). At the present time, it is difficult to explain why duplicating 7 amino acids in the neck improves processivity. For myosin, a long α-helix in the neck is thought to act as a lever arm to swing the motor between binding sites (Block, 1996), and changes in the length of this helix correlate with changes in the velocity of the motor (Uyeda, et al., 1996). However, as shown in this and previous studies (Inoue, et al., 1997), deletion of the kinesin neck coiled-coil does not significantly diminish the velocity of

movement, which argues that the kinesin neck helix does not act as a lever arm essential for movement, as is generally envisioned for myosin (Vale, 1996). It remains possible, however, that the neck helix increases processivity by amplifying mechanical changes that occur in the motor domain to increase the chance that the partner head will be reach and bind to the next tubulin dimer. Alternatively, if the 7 duplicated amino acids do not form part of the coiled-coil structure, they might enhance processivity simply by increasing the length of the linker between the two heads. However, this idea is inconsistent with our observation that addition of three glycines residues in the same location produces the opposite effect.

Location of processivity elements in kinesin

Our results, in conjunction with previous studies (Berliner, et al., 1995; Vale, et al., 1996), indicate that processivity requires a kinesin dimer and is optimized when the heads are connected by the neck coiled-coil. However, this work also eliminates the neck coiled-coil as a primary determinant for single motor motility, which implies that the catalytic domain and/or the neck β-sheet region contain important elements for processivity. This conclusion is consistent with the results of Case et al. (1997) and Henningsen et al. (1997), who found that the neck and stalk of kinesin are not sufficient to confer processivity on the catalytic domain of NCD, a kinesin superfamily member. Further mutagenesis studies in conjunction with single molecule assays can be used to better define the essential regions of kinesin that drive processive motion.

Acknowledgments

We wish to thank Nora Hom-Booher and Cindy Hart who provided valuable assistance with cloning. We also extend thanks to Roger Cooke for helpful discussion, Ryan Case and Guenther Woehlke for their comments on this manuscript, and to Dr. Eckhard Mandelkow for sharing unpublished data. L.R. received a Chancellor's graduate student fellowship from the University of California, San Francisco, and D.P. received postdoctoral support from the Jane Coffin Childs Foundation.

TABLE I

Motility and enzymatic characterization of neck mutant constructs

Kinesin Construct	Microtubule Gliding Speed[a] (μm/s)	ATPase[b]	
		k_{cat} (ATP/s/head)	K_m(MT) (μM Tubulin)
WT	0.32 ± 0.08	22 ± 3	0.97 ± 0.26
DEL	0.14 ± 0.02	15 ± 3	0.87 ± 0.12
GLY_3	0.23 ± 0.04	17 ± 3	1.36 ± 0.28
DUP	0.21 ± 0.04	16 ± 6	0.30 ± 0.07
YEN→ILI	0.14 ± 0.04	19 ± 3	0.85 ± 0.22
STABLE COIL	0.17 ± 0.04	31 ± 5	0.96 ± 0.59

Microtubules gliding on kinesin-coated surfaces and microtubule-stimulated ATPase assays were performed as described in "Materials and methods". Values are as follows:

a) Means ± S.D. of velocities of >60 microtubules were measured from assays with at least 2 independent protein preparations.

b) Means ± S.D. of at least four independent assays were performed on two different protein preparations. k_{cat} and K_m(MT) values were obtained by the best fit to a hyperbolic curve of 10-15 turnover rates at varying microtubule concentrations.

TABLE II

Motility of GFP-Kinesin constructs

K560-GFP Construct	Velocity[a] (μm/s)		Run Length (μm)		$P_{off/step}$[d] (%)	Movement Frequency[e]
	many motors	single motors	Observed[b]	Corrected[c]		movements / min•μm axoneme•nM kinesin
WT	0.40 ± 0.05	0.31 ± 0.07	0.93 ± 0.07	1.33	0.60	0.05
DEL	0.19 ± 0.07	0.17 ± 0.07	0.13 ± 0.01	0.14	5.6	0.05
GLY_3	0.28 ± 0.06	0.16 ± 0.09	0.38 ± 0.02	0.50	1.6	0.04
DUP	0.46 ± 0.1	0.31 ± 0.07	1.81 ± 0.17	4.35	0.18	0.07
			3.00 ± 0.43*	4.89*	0.16*	
YEN→ILI	0.28 ± 0.05	0.32 ± 0.08	0.83 ± 0.06	1.12	0.71	0.06
STABLE COIL	0.28 ± 0.04	0.29 ± 0.09	0.58 ± 0.04	0.73	1.1	0.37

Table II: Motile Properties of Kinesin-GFP Proteins

Microtubules gliding on kinesin-GFP coated surfaces and single molecule assays for kinesin-GFP using a total internal reflection microscope were performed as described in the Materials and Methods. Determination of the values in the Table were performed as described below:

a) Means and standard deviations were derived from measurements from 2 independent protein preparations. For microtubule gliding assays, >30 microtubule measurements were made per protein preparation. For single motor assays, >70 measurements were made per preparation and velocity data was derived only from those molecules that moved >0.2 μm.

b) The data from Figure 3 was fit to an exponential curve using the equation $y = A * e^{(-x/\lambda)}$, where λ = the average run length. Errors listed are the 95% confidence limits. Assays were performed using a laser power of 10 mW except where indicated by the asterisk (*) for the DUP construct, in which case 4 mW laser power was used.

c) In order to account for the photobleaching of GFP, the rate constant for release of kinesin from the microtubule (k_{rel}) was calculated according to the equation $k_{obs} = k_{rel} + k_{bleach}$, where k_{obs} = single motor velocity/observed run length, and k_{bleach} is the rate constant for bleaching of GFP-kinesin under a given laser power (0.1 s^{-1} for 10 mW and 0.04 s^{-1} for 4 mW (Pierce, et al., 1997; Pierce and Vale, in press). The corrected run length = velocity/k_{rel} (See Materials and Methods).

d) The probability that a kinesin will release from the microtubule rather than completing its next step was calculated using the equation $P_{off}/step = 1 - e^{(-s/\lambda)}$, where s is 8 nm (the kinesin step size, Svoboda, et al., 1993) and λ is the average run length.

e) For calculating the protein activity level, the total number of movements was divided by the total length of axonemes, time of observation, and the kinesin concentration. Since the percentage of runs which are <0.2 μm depends on the processivity of the mutant being measured, and the efficiency of detecting these runs is low, the total number of movements was derived by integrating the exponential curve fit to the data in Figure 3. The values listed are derived from the combined data from two preparations of the same mutant protein; when quantitated independently, assay to assay variability could be as great as two to three fold.

Figure 1: Mutant neck constructs

a) WT K560: The wild type kinesin construct used as the basis for mutagenesis contains the first 560 amino acids of the human kinesin gene followed by a histidine tag. * indicates residues at the hydrophobic interface of the coiled-coil. (*) indicates destabilizing residues in this interface. Domains of this construct are I) core catalytic domain, II) neck β-sheet region, III) neck coiled-coil region, IV) hinge region, V) coiled-coil stalk. The boundaries of the core catalytic domain are defined by conservation throughout the kinesin superfamily. The boundary of the neck is defined by strong class-specific conservation between conventional kinesins (Vale and Fletterick, 1997). b) DEL: Amino acids 341-370 of the neck coiled-coil were deleted. c) GLY3: Three glycines were inserted between K342 and K343. d) DUP: Residues T336-K342, one complete turn of the α-helix, were duplicated. e) YEN→ILI: Three destabilizing residues at the "a" and "d" position of the coiled-coil were changed to stabilizing hydrophobic residues (Y344I, E347L, and N351I). f) STABLE COIL: Four heptad repeats (a.a. 343-370), were replaced by a highly stable model coiled-coil consisting of 4 repeats of the sequence EIEALKA. g) WT K560-GFP: The above neck mutations (b-f) were also inserted into K560 with green fluorescent protein (the Ser65Thr mutant, Heim, et al., 1995) fused to its C-terminus.

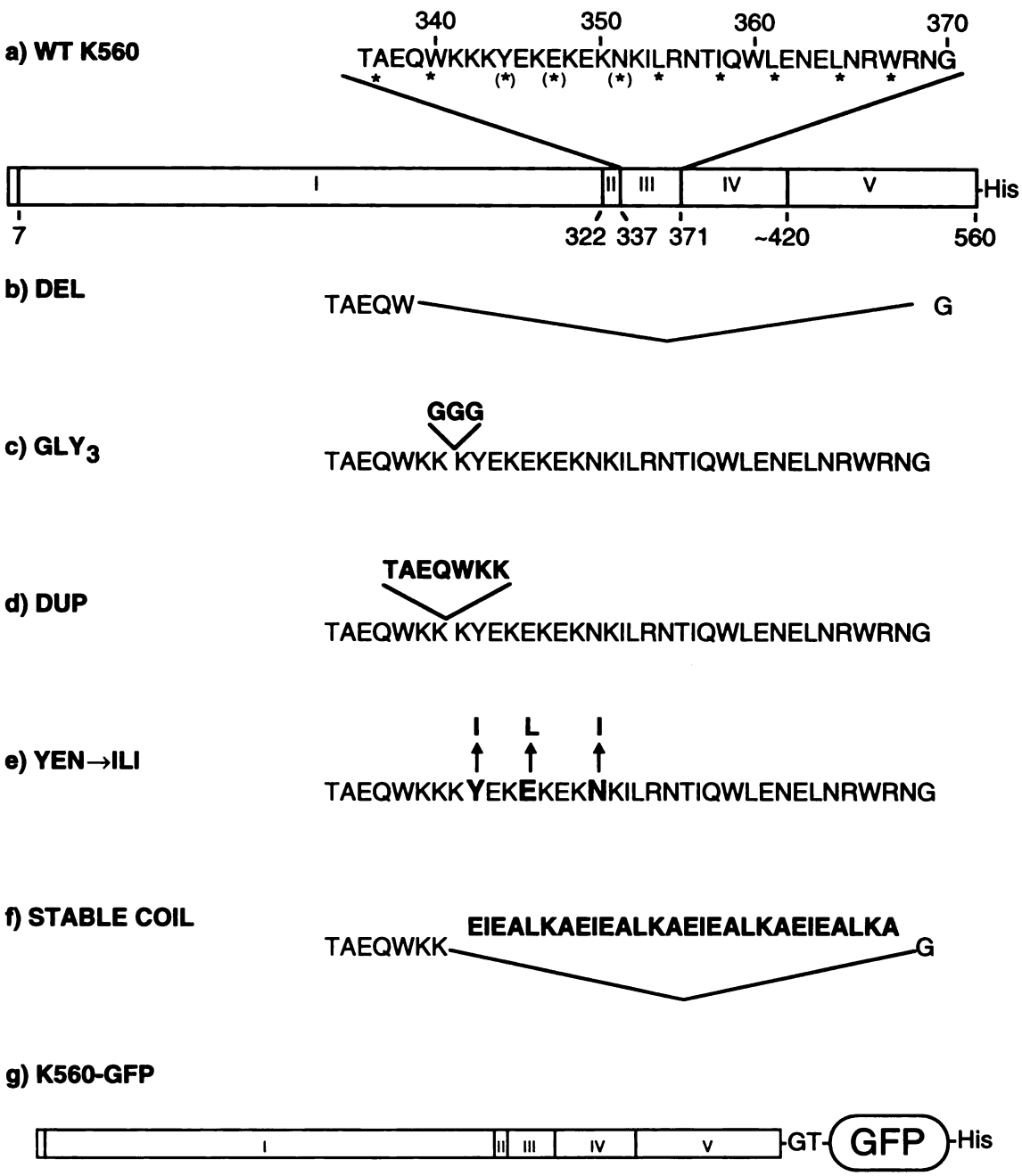

a) WT K560
340
350
360
370
TAEQWKKKYEKEKEKNKILRNTIQWLENELNRWRNG
I
II
III
IV
V
His
7
322
337
371
~420
560
b) DEL
TAEQW
G
c) GLY3
GGG
TAEQWKK KYEKEKEKNKILRNTIQWLENELNRWRNG
d) DUP
TAEQWKK
TAEQWKK KYEKEKEKNKILRNTIQWLENELNRWRNG
e) YEN→ILI
I L I
TAEQWKKK YEK EKEK NKILRNTIQWLENELNRWRNG
f) STABLE COIL
EIEALKAEIEALKAEIEALKAEIEALKA
TAEQWKK
G
g) K560-GFP
I
II
III
IV
V
GT-
GFP
-His

Figure 2: Fluorescent intensity of individual kinesin molecules

The histograms show the fluorescent intensity of kinesin molecules either moving along axonemes or non-specifically adsorbed onto the slide surface nearby (see Materials and Methods for details). Tick marks represent one arbitrary fluorescent unit; fluorescent intensities cannot be directly compared between preparations because of small variations in laser alignment during different assays.

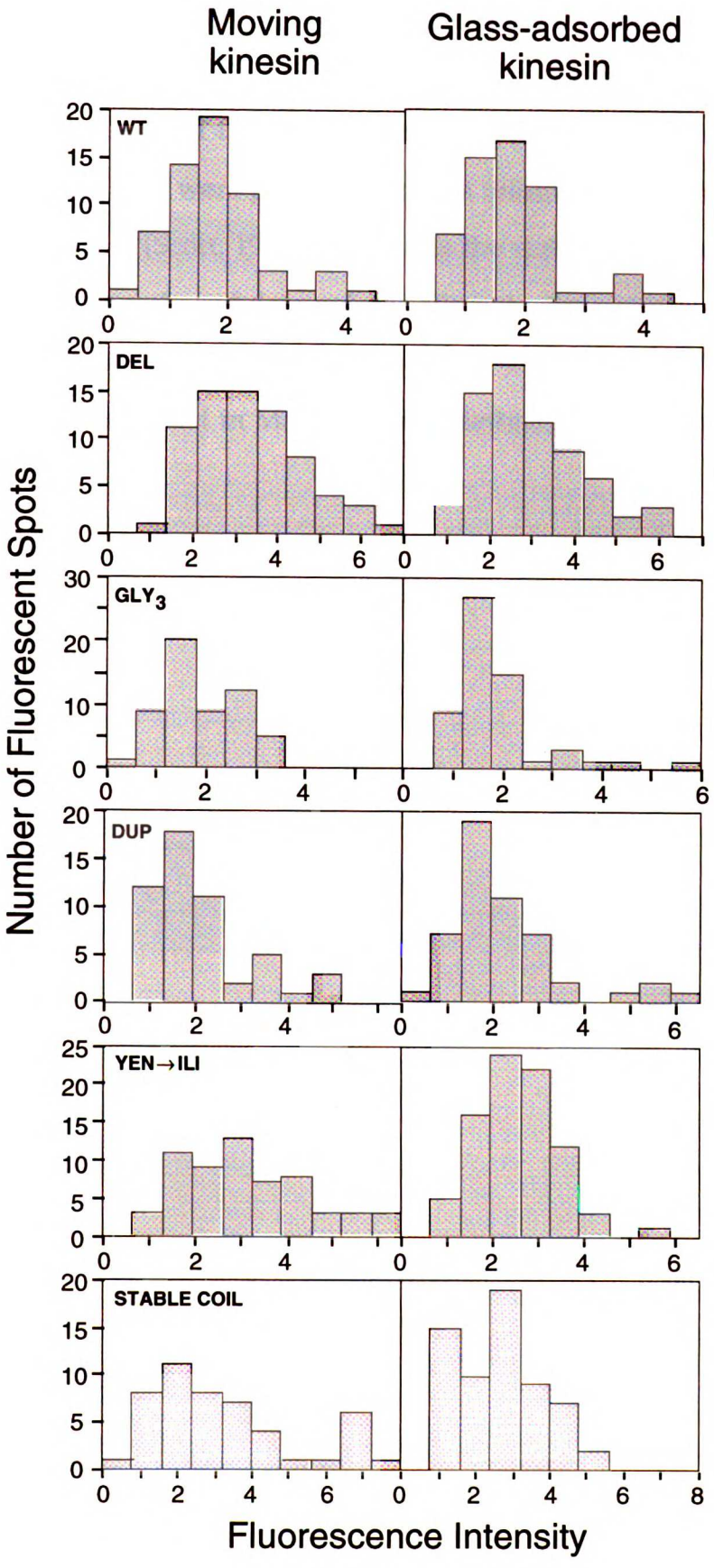
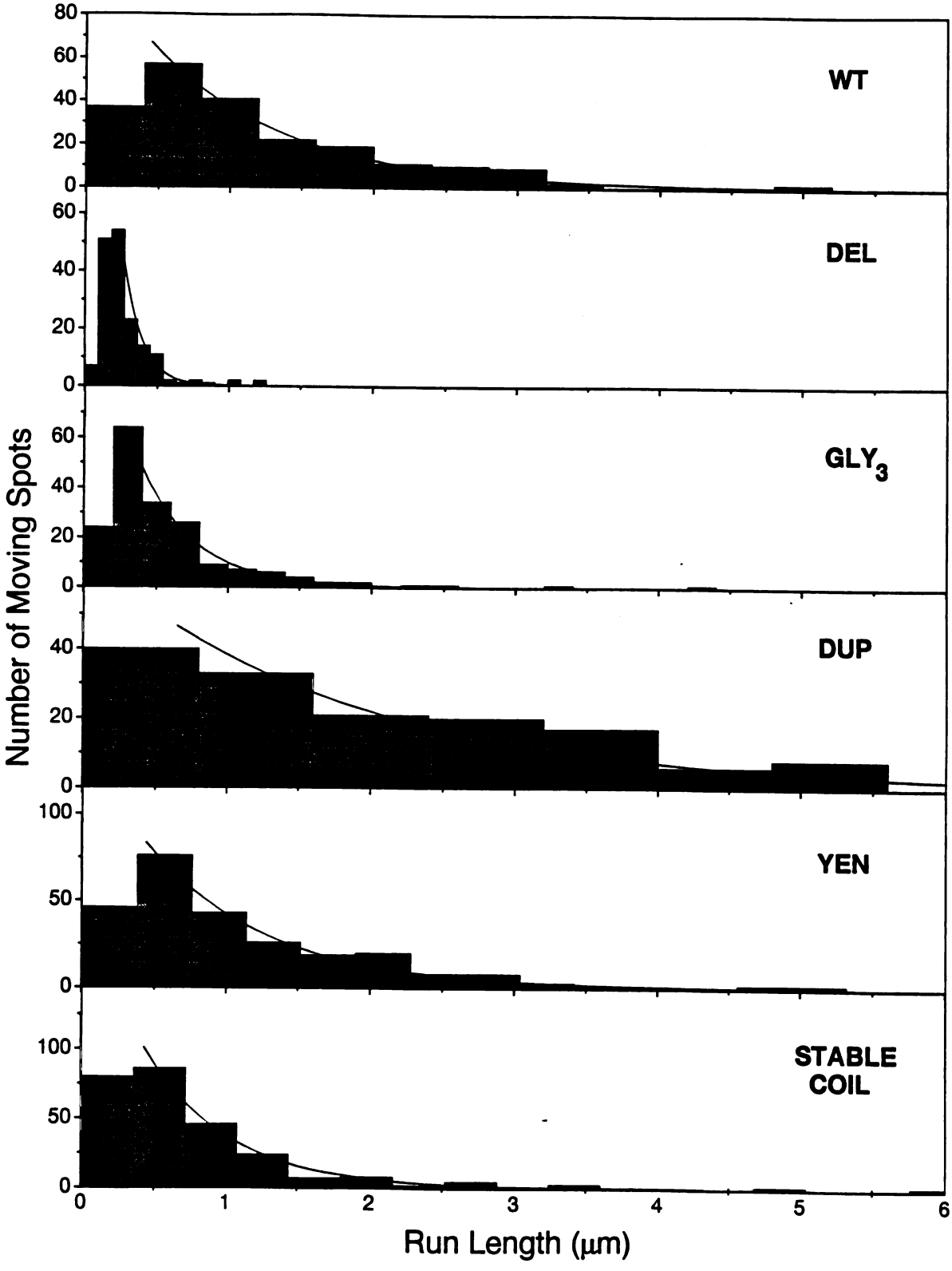

Moving kinesin
Glass-adsorbed kinesin
WT
DEL
GLY3
DUP
YEN→ILI
STABLE COIL
Number of Fluorescent Spots
Fluorescence Intensity

Figure 3: Run lengths of single, fluorescently labeled kinesin molecules

Run lengths of 150-270 individual GFP-kinesin molecules moving on axonemes were measured from 2 independent preparations of each construct. Histograms of the data were plotted using bin widths derived from the formula $2.6\sigma N^{(-1/3)}$ (Scott, 1979), where σ is the standard deviation of the data and N is the number of data points collected. Exponential curves were fit to the data using only runs >0.25 μm (or 0.2 and 0.15 μm for GLY_3 and DEL respectively), as described in Materials and Methods. Run length values are shown in Table 2.


WT
DEL
GLY3
DUP
YEN
STABLE COIL
Number of Moving Spots
Run Length (μm)
0
20
40
60
80
50
100
1
2
3
4
5
6

CONCLUSION

Seven years ago, when this thesis began, it was known that individual kinesin molecules could use the energy from ATP hydrolysis to move processively towards the plus end of microtubules (Block, et al., 1990; Howard, et al., 1989; Vale, et al., 1985; Vale, et al., 1985). However, the mechanism underlying this movement was not yet understood. A complete explanation of kinesin motility must be able to describe how the motor couples three basic processes: ATP hydrolysis, reversible binding to the filament, and directional positioning relative to the polymer lattice. Studies of these properties have often been complicated by the existence of interactions between the two heads of the dimer. Below, I will consider what has been determined so far about these three properties in kinesin, how they are influenced by dimerization, and what areas remain for future research.

The ATP hydrolysis cycle

Kinetic experiments have extensively mapped out kinesin's ATP hydrolysis cycle (for review, see Hackney, 1996). Almost all the rates of transitions between intermediates have been measured for both the monomer and the dimer. In the absence of microtubules, the two heads act independently and the hydrolysis rate is limited by extremely slow ADP release. In the presence of microtubules, ADP release is accelerated almost 10,000-fold, although it still may be partially rate limiting during the cycle. The strongest evidence so far for an interaction between the two kinesin heads has come from kinetic experiments showing that microtubules can only accelerate the release of ADP from one head of a dimer at a time.

The structural basis for these kinetic transition rates is not yet understood. An atomic structure of the nucleotide binding pocket is known for only one form of kinesin, that bound to ADP (Kull, et al., 1996). However,

because kinesin's nucleotide binding pocket is similar to that of myosin and G-proteins, analogous amino acids in all three proteins may be sensing the presence of the gamma phosphate or controlling rate of ADP release (Vale, 1996). In particular, two conserved residues in G proteins interact with the gamma phosphate and act as switches to change the properties of the protein when it is bound to ATP versus ADP. Although mutagenesis studies can explore the function of the corresponding amino acids in kinesin, a detailed understanding of these interactions will probably await the crystallization of the protein in different nucleotide states.

Microtubule Binding

Experiments have begun to yield insight into kinesin's microtubule binding properties, although the information here is less complete than for motor's ATPase cycle. The affinity of kinesin for the microtubule has been measured for almost all hydrolysis intermediates except kinesin·ADP·Pi (Crevel, et al., 1996; Ma and Taylor, 1995; Romberg and Vale, 1993). In addition, residues in the microtubule binding site have been defined through alanine scanning mutagenesis (Woehlke, et al., 1997). However, this technique probably only detected mutations that severely altered microtubule affinity during the weak binding kinesin·ADP intermediate. Therefore, additional residues remain to be discovered that strengthen binding during other parts of the hydrolysis cycle or that have more subtle effects. Once the residues that modulate binding are understood, models for communication between the microtubule binding site and the nucleotide pocket can begin to be seriously considered.

A picture of the tubulin side of this binding interaction is also starting to take shape. Kinesin binds once per tubulin dimer, largely to β tubulin,

with some contacts on the alpha subunit (Larcher, et al., 1996; Song and Mandelkow, 1993; Tucker and Goldstein, 1997; Walker, 1995). Some controversy exists as to whether dimeric kinesin binds to a single tubulin dimer or can straddle two dimers with its two motor domains (Arnal, et al., 1996; Hirose, et al., 1996). Although no conclusive evidence for a kinesin with both heads bound has yet been found, such an intermediate needs to exist at least transiently to explain the motor's processivity. An exciting recent development is the solution of tubulin's atomic structure. Combined with EM images that allow the kinesin structure to be docked onto the microtubule, this new information should allow the tubulin residues involved in kinesin binding to be determined. In addition, EM pictures have indicated that tubulin changes conformations upon kinesin binding (Hoenger, et al., 1995), and it will be interesting to see if these changes in turn influence kinesin function.

The powerstroke and directional movement

The biggest gap in our understanding of kinesin function is information on how the motor moves directionally. Models for kinesin's powerstroke (Cordova, et al., 1992; Hirose, et al., 1996; Howard, 1996; Vale, 1996; Young, et al., 1995) can be divided into two categories that are not necessarily mutually exclusive. The models describe either a lever arm or an elastic spring moving the motor between binding sites on the microtubule (Figure 1, Introduction). In the first type of model, a conformational change causes a region of the protein to swing as a rigid body along the microtubule. After the motor has bound to the filament, displacement occurs as the motor's lever arm returns to its original conformation. In the second type of model, a structured region of the motor extends or becomes more flexible.

This extension allows the motor to perform a diffusional search to find a new binding site. Following tubulin binding, the reformation of the original structure acts like a spring to pull the filament towards the motor. For many years, these same models were considered in discussions of the myosin powerstroke, and only recently have high resolution structures of myosin provided evidence that a long α helix may act as a lever arm to swing the motor between actin subunits (Jontes, et al., 1996; Whittaker, et al., 1996).

For kinesin, it is unknown which portion of the protein is involved in producing the powerstroke. The region involved must also be the region that determines the direction of movement for kinesin and for NCD, a minus-end directed, kinesin-like protein. No obvious lever arms exist in the conserved motor core of the kinesin superfamily (the catalytic core), which forms a single, compact domain (Kull, et al., 1996; Sablin, et al., 1996). However, proteins consisting of the catalytic core plus ~10 residues of the neighboring neck domain can produce unidirectional movement (Stewart, et al., 1993) in both kinesin and NCD. Because the neck sequence is specific to subclasses of kinesin-like proteins, this region is a good candidate for producing the powerstrokes that move these proteins in different directions. The importance of the neck to directionality has been supported by recent experiments showing that the kinesin neck can confer plus-ended directionality on the NCD catalytic core (Case, et al., 1997; Henningsen and Schliwa, 1997).

Studies have provided evidence that kinesin's powerstroke may occur during ATP binding (Ma and Taylor, 1997) or following hydrolysis (Higuchi, et al., 1997) (n.b. force production may occur during different intermediates in the hydrolysis cycle for monomers and dimers, see Howard, 1996). Therefore, for the neck to be involved in the motor's powerstroke, the neck structure

should change during the appropriate nucleotide transitions. The possibility of such a transition is supported by different crystal structures of the motors that show the neck either bound to the catalytic core of the motor or completely disordered (Kull, et al., 1996; Sablin, et al., 1996, Mandelkow, submitted), suggesting that the neck-head interaction may be labile.

How could the neck produce the motor's powerstroke? There is some evidence that the neck may not be acting as a rigid lever arm during motility (Inoue, et al., 1997). However, simple melting of the neck to form a flexible polypeptide tether is also insufficient to explain directional motion that differs between kinesin and NCD. In the structures of both motors, the neck emerges from the same region of the protein and is similarly oriented relative to the head (Sablin, personal communication, Mandelkow, submitted). Therefore, in order for the necks to be essential to directionality, some as yet unseen structural intermediate needs to exist in which the necks orient kinesin and NCD towards opposite ends of the microtubule.

Many models for directionality have been based on EM images of the dimeric motors (Arnal, et al., 1996; Hirose, et al., 1996). In these images, one head is bound to the microtubule and is superimposable between the kinesin and NCD structures, while the second head is unbound and oriented differently in the two proteins. It is important to remember, however, that truncated monomers retain their native direction of movement, meaning that interactions between the two heads of a dimer are not essential to directionality. Since those amino acids in the necks that are necessary for directionality are oriented identically in recent crystal structures, perhaps the suggestive difference between kinesin and NCD dimers that appears in the EM images are not actually important to determining directionality. Another

possibility is that the structure of the necks in the EM images is not the same as that seen in the crystal structures.

Processivity

Kinesin's dimeric structure, while not necessary for directionality, is essential for single motor motility. For kinesin to be processive, every power stroke must place the motor 8 nm further along the microtubule. Individual motor domains are only ~7 nm on their longest axis (Kull, et al., 1996), and thus dimerization is essential to extending the reach of the protein. However, simply connecting two motor domains is not enough to make them processive (Case, et al., 1997). The power stroke in one head must be amplified by the second head, which may act as an elongated tether or lever arm. In addition, the two heads must synchronize their chemomechanical cycles.

The region of kinesin that coordinates the two heads remains to be defined (see chapter 3). A 33 residue coiled-coil in the kinesin neck allows dimerization of the motor, and is therefore positioned to be a potential communication link between the heads. However, deletion of 29 of these residues (a.a. 341-370) results in a motor that is still able to step between tubulin dimers with a high efficiency. The four residues that are not altered in this deletion construct may contribute to processivity, a possibility that is supported by results with the DUP construct, in which duplication of a.a. 336-342 enhanced processivity above that seen in wild type kinesin. In order to determine whether these initial amino acids are essential to processivity, the entire neck coiled-coil (a.a. 337-370) must be deleted.

Modifications of the neck that are based on the results with the DUP construct may provide the easiest handle for further defining the features that

are important for processivity. To determine whether length of the neck is correlated with single motor run lengths, the processivity of a series of proteins in which a.a. 336-342 are deleted or repeated multiple times could be analyzed. To distinguish the importance of the length of the neck from properties specific to the N-terminal residues of the coiled-coil, a.a. 343-349 could instead be duplicated or deleted from the wild type or the DUP construct. In addition, mutations that stabilize the initial heptad against unwinding could be made to determine whether this region needs to unfold during single motor motility.

If the neck coiled-coil aligns the two heads so that they can readily interact with each other, insertions or deletions in this region would need to occur in multiples of 7 amino acids in order not to disturb the phasing of the coil. However, it is possible that in DUP, the replicated amino acids form an unstructured, flexible linker. By introducing insertions that were not multiples of 7 residues, or by scrambling the first heptad in DUP, one could determine whether the structure rather than the length or charge of the duplicated peptide is important to enhancing processivity.

Kinetic studies of the DUP protein's interaction with microtubules may provide more detailed insight into the mechanism of single molecule motility. In DUP, a longer linker may allow the free head of a dimer to bind more rapidly to the next tubulin dimer before the original head has released from the microtubule. As a result, the motor might spend more time with both heads on the filament. Such changes in binding of the heads to the microtubule should be detectable in experiments examining either the rate of release of ADP from the two heads of a dimer (as in Hackney, 1994), or the percentage of time the motor spends with both heads bound to the microtubule.

In addition to residues in the neck coiled-coil, sequences within either the β-strand portion of the neck or the catalytic core must also contribute to the reversible connection between the motor domains (Case, et al., 1997). Images of kinesin dimers show that the cores contact each other at the tip of their arrow head-shaped structures (Sosa, et al., 1997, Mandelkow, submitted). In the crystal structure of the kinesin dimer, the neck β-strands are located at the edge of the central β-sheet of the separate head domains. However, this sequence may also be able to dimerize with itself, since peptides of the entire neck region (β-strand + coiled-coil) form more stable dimers than those which exclude the β-strand region (Tripet, et al., 1997). To be involved in processivity, dimerizing interactions in the catalytic core or the β-sheet of the neck should be nucleotide sensitive and transiently denatured in order to allow both heads to bind to the microtubule.

Mutagenesis studies of the essential neck β-sheet or core domains of kinesin would need to be carefully directed towards those residues that are likely to be specifically involved in processivity. By substituting residues in the neck β-sheet that are conserved in conventional kinesins with those which are found in other N-terminal motors, one might affect processivity while preserving the basic motility of the protein. Similarly, mutations in residues involved in the contact between the catalytic cores will be less likely to disrupt general motor function if they are on the surface of the motor and conserved in kinesin but not in the entire superfamily.

For other motors within the kinesin superfamily, do the two heads interact? If the heads are loosely connected, there may be no steric hindrance to force them to alternate. In addition, other motors would not be processive if the heads do not amplify each other's motion enough to span the full 8 nm between binding sites. Although no motor besides conventional kinesin has

yet been shown to be processive, all proteins that move organelles should face similar evolutionary pressures for efficient motility driven by only a few motor molecules. If in the future other motors are not found to be processive, perhaps the *in vivo* conditions under which motors work enhance their processivity in ways that differ from *in vitro* motility assays. Alternatively, conventional kinesin may be adapted for moving especially small cellular components such as protein complexes.

References

Amos, L. A. and K. Hirose. 1997. The structure of microtubule-motor complexes. *Curr. Opin. Cell Biol.* 9:4-11.

Arnal, I., F. Metoz, S. DeBonis and R. H. Wade. 1996. Three-dimensional structure of functional motor proteins on microtubules. *Curr. Biol.* 6:1265-1270.

Ashkin, A., K. Schutze, J. M. Dziedzic, U. Euteneuer and M. Schliwa. 1990. Force generation of organelle transport measured in vivo by an infrared laser trap [see comments] . *Nature.* 348:346-8.

Bagshaw, C. R. 1993. Muscle Contraction.

Berliner, E., E. C. Young, K. Anderson, H. Mahtani and J. Gelles. 1995. Failure of a single-headed kinesin to track parallel to microtubule protofilaments. *Nature.* 373:718-721.

Block, S. M. 1996. Fifty ways to love your lever: myosin motors. *Cell.* 87:151-157.

Block, S. M., L. S. Goldstein and B. J. Schnapp. 1990. Bead movement by single kinesin molecules with optical tweezers. *Nature.* 348:348-352.

Bloom, G. and S. Endow. 1995. Motor Proteins 1: kinesin. *Protein Profile.* 1112-1138.

Case, R. B., D. W. Pierce, N. Hom-Booher, C. L. Hart and R. D. Vale. 1997. The directional preference of kinesin motors is specified by an element outside of the motor catalytic domain. *Cell.* 90:959-966.

Catterall, W. A. and P. L. Pederson. 1971. *J. Biol. Chem.* 246:4987-4994.

Chamberlin, M. 1995. New models for the mechanism of transcription elongation and its regulation. *Harvey Lect.* 88:1-21.

Cooke, R. 1997. Actomyosin interaction in striated muscle. *Physiological Reviews*. 77:671-697.

Cooke, R., M. S. Crowder and D. D. Thomas. 1982. Orientation of spin labels attached to cross-bridges in contracting muscle fibres. 300:776-778.

Coppin, C., J. Finer, J. S. Spudich and R. D. Vale. 1995. Measurement of the isometric force exerted by a single kinesin molecule. *Biophys. J.* 68:242s-244s.

Cordova, N. J., B. Ermentrout and G. F. Oster. 1992. Dynamics of single-motor molecules: the thermal ratchet model. . *Proc Natl Acad Sci U S A.* 89:339-43.

Correia, J. J., S. P. Gilbert, M. L. Moyer and K. A. Johnson. 1995. Sedimentation studies on the kinesin motor domain constructs K401, K366, and K341. *Biochemistry*. 34:4898-4907.

Crevel, I. M.-T. C., A. Lockhart and R. A. Cross. 1996. Weak and strong states of kinesin and ncd. *J. Mol. Biol.* 257:66-76.

Finer, J. T., R. M. Simmons and J. A. Spudich. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature*. 368:113-119.

Funatsu, T., Y. Harada, M. Tokunaga, K. Saito and Y. Yanagida. 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature*. 374:555-559.

Gibbons, I. R. 1996. The role of dynein in microtubule-based motility. *Cell structure and Function*. 21:331-42.

Gibbons, I. R. and E. Fronk. 1979. A latent adenosine triphosphatase form of dynein 1 from sea urchin sperm flagella. *J. Biol. Chem.* 254:187-196.

Gilbert, S. P., M. R. Webb, M. Brune and K. A. Johnson. 1995. Pathway of processive ATP hydrolysis by kinesin. *Nature*. 373:671-676.

Goldstein, L. S. 1993. With apologies to Scheherazade: tails of 1001 kinesin motors. *Ann. Rev. Genetics.* 27:319-351.

Guzman, C. A., M. J. Walker, M. Rohde and K. N. Timmis. 1992. Expression of Bordetella pertussis filamentous hemagglutinin in Echerichia coli using a two cistron system. *Microbial Pathogenesis.* 12:383-389.

Hackney, D. D. 1988. Kinesin ATPase: rate-limiting ADP release.. *Proc. Natl. Acad. Sci. U.S.A.* 85:6314-6318.

Hackney, D. D. 1992. Kinesin and myosin ATPases: variations on a theme.. *Philos Trans R Soc Lond Biol.* 336:13-7.

Hackney, D. D., J. D. Levitt and J. Suhan. 1992. Kinesin undergoes a 9 S to 6 S conformational transition.. *J Biol Chem.* 267:8696-701.

Hackney, D. D. 1994. Evidence for alternating head catalysis by kinesin during microtubule-stimulated ATP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* 91:6865-6869.

Hackney, D. D. 1995. Highly processive microtubule-stimulated ATP hydrolysis by dimeric kinesin head domains. *Nature.* 377:448-450.

Hackney, D. D. 1996. The kinetic cycles of myosin, kinesin, and dynein. *Annu. Rev. Physiol.* 58:731-750.

Hackney, D. D. and T.-G. Huang. 1993. Interaction of kinesin head domains with tail domains. *Molecular Biology of the Cell.* 4:49.

Hackney, D. D., A. S. Malik and K. W. Wright. 1989. Nucleotide-free kinesin hydrolyzes ATP with burst kinetics.. *J Biol Chem.* 264:15943-8.

Hancock, W. O. and J. Howard. 1996. Motility of Single Headed Kinesin. *Molecular Biology of the Cell.* 7, supp.:214a.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol. Biol.* 216:49-68.

Heim, R., A. B. Cubitt and R. Y. Tsien. 1995. Improved green fluorescence. *Nature.* 373:663-664.

Henningsen, U. and M. Schliwa. 1997. Reversal in the direction of movement of a molecular motor. *Nature.* 389:93-95.

Higuchi, H., E. Muto, Y. Inoue and T. Yanagida. 1997. Kinetics of force generation by single kinesin molecules activated by laser photolysis of caged ATP. *Proc. Natl. Acad. Sci. U.S.A.* In press:

Hirose, K., A. Lockhart, R. A. Cross and L. A. Amos. 1996. Three-dimensional cryoelectron microscopy of dimeric kinesin and ncd motor domains on microtubules. *Proc. Natl. Acad. Sci. U.S.A.* 93:9539-9544.

Hoenger, A., E. P. Sablin, R. D. Vale, R. J. Fletterick and R. A. Milligan. 1995. Three-dimensional structure of a tubulin-motor-protein complex. *Nature.* 376:271-274.

Holde, K. E. v. and W. O. Weischet. 1978. Boundary Analysis of Sedimentation Velocity Experiments with Monodisperse and Paucidisperse Solutes. *Biopolymers.* 17:1387-1403.

Howard, J. 1996. The movement of kinesin along microtubules. *Annu. Rev. Physiol.* 58:703-729.

Howard, J. 1997. Molecular motors: structural adaptations to cellular functions. *Nature.* 389:561-567.

Howard, J., A. J. Hudspeth and R. D. Vale. 1989. Movement of microtubules by single kinesin molecules. *Nature.* 342:154-158.

Huang, T.-G. and D. D. Hackney. 1993. Drosophila kinesin minimal motor domain expressed in Escherichia coli: purification and kinetic characterization. *J. Biol. Chem.* 269:16493-16501.

Huang, T. G., J. Suhan and D. D. Hackney. 1994. Drosophila kinesin motor domain extending to amino acid position 392 is dimeric when expressed in Escherichia coli. *J. Biol. Chem.* 269:16502-16507.

Hunt, A. J. and J. Howard. 1993. Kinesin swivels to permit microtubule movement in any direction. *Proc. Natl. Acad. Sci. U.S.A.* 90:11653-11657.

Inoue, Y., Y. Y. Toyoshima, A. H. Iwane, S. Morimoto, H. Higuchi and T. Yanagida. 1997. Movements of truncated kinesin fragments with a short or an artificial flexible neck. *Proc. Natl. Acad. Sci.* 94:7275-7280.

Jiang, W. and D. D. Hackney. 1997. Monomeric kinesin head domains hydrolyze multiple ATP molecules before release from a microtubule. *The Journal of Biological Chemistry.* 272:5616-5621.

Jiang, W., M. F. Stock, X. Li and D. D. Hackney. 1997. Influence of the kinesin neck domain on dimerization and ATPase kinetics. *The Journal of Biological Chemistry.* 272:7626-7632.

Jontes, J. D., E. M. Wilson-Kubalek and R. A. Milligan. 1996. The brush border myosin-I tail swings through a 32° arc upon ADP release. *Nature.* 378:751-753.

Kull, F. J., E. P. Sablin, R. Lau, R. J. Fletterick and R. D. Vale. 1996. Crystal structure of the kinesin motor domain reveals a structural similarity to myosin. *Nature.* 380:550-555.

Kuznetsov, S. A. and V. I. Gelfand. 1986. Bovine brain kinesin is a microtubule-activated ATPase. . *Proc Natl Acad Sci U S A.* 83:8530-4.

Kuznetsov, S. A., E. A. Vaisberg, N. A. Shanina, N. N. Magretova, V. Y. Chernyak and V. I. Gelfand. 1988. The quaternary structure of bovine brain kinesin. . *Embo J.* 7:353-6.

Larcher, J. C., D. Boucher, S. Lazereg, F. Gros and P. Denoulet. 1996. Interaction of kinesin motor domains with alpha- and beta-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation. *Journal of BIological Chemistry*. 271:22117-22124.

Leibler, S. and D. A. Huse. 1993. Porters versus rowers: a unified stochastic model of motor proteins. *Journal of Cell Biology*. 121:1357-1368.
Lohman, T. M. and K. P. Bjornson. 1996. Mechanisms of helicase-catalyzed DNA unwinding. *Annual Review of Biochemistry*. 35:2268-2282.

Ma, Y.-Z. and E. W. Taylor. 1997. Interacting head mechanism of microtubule-kinesin ATPase. *J. Biol. Chem.* 272:724-730.

Ma, Y. Z. and E. W. Taylor. 1995. Mechanism of microtubule kinesin ATPase. *Biochemistry*. 34:13242-13251.

Ma, Y. Z. and E. W. Taylor. 1997. Kinetic mechanism of a monomeric kinesin construct. *Journal of Biological Chemistry*. 272:717-723.

Miller, R. H. and R. J. Lasek. 1985. Crossbridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181-2193.

Mooseker, M. S. and R. E. Cheney. 1995. Unconventional myosin. *Annu. Rev. Cell Biol.* 11:633-675.

Morii, H., T. Takenawa, F. Arisaka and T. Shimizu. 1997. Identification of kinesin neck region as a stable α-helical coiled-coil and its thermodynamic characterization. *Biochemistry*. in press:

Pierce, D. W., N. Hom-Booher and R. D. Vale. 1997. Imaging individual green fluorescent proteins. *Nature*. 388:338.

Pierce, D. W. and R. D. Vale. in press. Visualization of single GFP molecules and applications to assaying single protein dynamics. *Methods in Cell Biology*.

Porter, M. E. 1996. Axonemal dyneins: assembly, organization, and regulation. *Current Opinion in Cell Biology*. 8:10-17.

Rayment, I., W. R. Rypniewski, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg and H. M. Holden. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science*. 261:50-58.

Rayment, I., C. Smith and R. G. Yount. 1996. The active site of myosin. *Annual Review of Physiology*. 58:671-702.

Romberg, L. and R. D. Vale. 1993. Chemomechanical cycle of kinesin differs from that of myosin. *Nature*. 361:168-170.

Sablin, E. P., F. J. Kull, R. Cooke, R. D. Vale and R. J. Fletterick. 1996. Crystal structure of the motor domain of the kinesin-related motor ncd. *Nature*. 380:555-559.

Schnapp, B. J., B. Crise, M. P. Sheetz, T. S. Reese and S. Kahn. 1990. Delayed start-up of kinesin-driven microtubule gliding following inhibition by adenosine 5'-(b,g-imido)triphosphate. *Proc. Natl. Acad. Sci. U.S.A.* 87:10053-10057.

Schoner, B. E., R. M. Belagaje and R. G. Schoner. 1986. Translation of a synthetic two-cistron mRNA in Escherichia coli. *Proc. Natl. Acad. Sci. USA*. 83:8506-8510.

Scott, D. W. 1979. On optimal and data-based histograms. *Biometrika*. 66:605-610.

Song, Y. H. and E. Mandelkow. 1993. Recombinant kinesin motor domain binds to beta-tubulin and decorates microtubules with a B surface lattice. . *Proc Natl Acad Sci U S A.* 90:1671-5.

Sosa, H., P. Dias, A. Hoenger, M. Whittaker, E. Wilson-Kubalek, E. Sablin, R. J. Fletterick, R. D. Vale and R. A. Milligan. 1997. A model for the microtubule-ncd motor protein complex obtained by cryo-electron microscopy and image analysis. *Cell.* 90:217-224.

Spudich, J. A. 1990. Motor molecules in motion. *Nature.* 348:284-285.

Stewart, R. J., J. P. Thaler and L. S. Goldstein. 1993. Direction of microtubule movement is an intrinsic property of the motor domains of kinesin heavy chain and Drosophila ncd protein. *Proc. Natl. Acad. Sci. U. S. A.* 90:5209-5213.

Su, J. Y., R. S. Hodges and C. M. Kay. 1994. Effect of chain length on the formation and stability of synthetic alpha-helical coiled-coils. *Biochemistry.* 33:15501-15510.

Svoboda, K., C. F. Schmidt, B. J. Schnapp and S. M. Block. 1993. Direct observation of kinesin stepping by optical trapping interferometry. *Nature.* 365:721-727.

Tripet, B., R. D. Vale and R. S. Hodges. 1997. Demonstration of coiled-coil interactions within the kinesin neck region using synthetic peptides: implications for motor activity. *J. Biol. Chem.* in press:

Tucker, C. and L. S. B. Goldstein. 1997. Probing the kinesin-microtubule interaction. *J. Biol. Chem.* 272:in press.

Uyeda, T. Q., S. J. Kron and J. A. Spudich. 1990. Myosin step size. Estimation from slow sliding movement of actin over low densities of heavy meromyosin. *J. Mol. Biol.* 214:699-710.

Uyeda, T. Q. P., P. D. Abramson and J. A. Spudich. 1996. The neck region of the myosin motor domain acts as a lever arm to generate movement. *Proc. Natl. Acad. Sci. U.S.A.* 93:4459-4464.

Vale, R. D. 1992. Microtubule motors: many new models off the assembly line. *Trends Biochem. Sci.* 17:300-304.

Vale, R. D. 1996. Switches, latches, and amplifiers: common themes of molecular motors and G proteins. *J. Cell Biol.* 135:291-302.

Vale, R. D. and R. J. Fletterick. 1997. The design plan of kinesin motors. *Annu. Rev. Cell Dev. Biol.* 12:745-777.

Vale, R. D., T. Funatsu, D. W. Pierce, L. Romberg, Y. Harada and T. Yanagida. 1996. Direct observation of single kinesin molecules moving along microtubules. *Nature.* 380:451-453.

Vale, R. D. and F. Oosawa. 1990. Protein motors and Maxwell's demons: does mechanochemical transduction involve a thermal ratchet? *Adv. Biophys.* 26:97-134.

Vale, R. D., B. J. Schnapp, T. Mitchison, E. Steuer, T. S. Reese and M. P. Sheetz. 1985. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. . *Cell.*

Vale, R. D., B. J. Schnapp, T. S. Reese and M. P. Sheetz. 1985. Purification and structure of a novel microtubule translocator (kinesin) from squid and bovine neural tissue. *J. Cell Biol.* 101:37a.

Vale, R. D., D. M. Soll and I. R. Gibbons. 1989. One-dimensional diffusion of microtubules bound to flagellar dynein. *Cell.* In press.

Walker, R. A. 1995. ncd and kinesin motor domains interact with both alpha- and beta-tubulin. *Proc. Natl. Acad. Sci. U. S. A.* 92:5960-5964.

Weiss, A. and L. A. Leinwand. 1996. The mammalian myosin heavy chain gene family. *Annual Review of Cell and Developmental BIology*. 12:417-439.

Whittaker, M., E. M. Wilson-Kubalek, J. E. Smith, L. Faust, R. A. Milligan and H. L. Sweeney. 1996. Smooth muscle myosin moves 35 Å upon ADP release. *Nature*. 378:748-751.

Woehlke, G., A. K. Ruby, C. L. Hart, B. Ly, N. Hom-Booher and R. D. Vale. 1997. Microtubule Interaction Site of the Kinesin Motor. *Cell*. 90:207-216.

Wyman, C. and M. Botchan. 1995. A familiar ring to DNA polymerase processivity. *Current Biology*. 5:334-337.

Young, E. C., E. Berliner, H. K. Mahtani, B. Perez-Ramirez and J. Gelles. 1995. Subunit interactions in dimeric kinesin heavy chain derivatives that lac the kinesin rod. *J. Biol. Chem.* 270:3926-3931.