THE TWO MOTOR DOMAINS OF KINESIN-2 COORDINATE FOR PROCESSIVE MOTILITY

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by
Yangrong Zhang

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The thesis of Yangrong Zhang was reviewed and approved* by the following:

William O. Hancock  
Assistant Professor of Bioengineering  
Thesis Advisor  
Chair of Committee  

Peter J. Butler  
Assistant Professor of Bioengineering  

Richard J. Cyr  
Professor of Biology  

Cheng Dong  
Professor of Bioengineering  

Ahmed A. Heikal  
Associate Professor of Bioengineering  

Herbert H. Lipowsky  
Professor of Bioengineering  
Head of the Department of Bioengineering  

*Signatures are on file in the Graduate School.
ABSTRACT

The long-term goal of this project is to understand the fundamental chemomechanical mechanism of the Kinesin-2 class of molecular motor proteins. Kinesin-2 motors, involved in intraflagellar transport and Golgi trafficking, are unique among the kinesin superfamily in having two non-identical motor domains. Because homodimeric kinesins rely on coordination between their two motor domains for long distance transport, this heterodimeric structure offers many potential functional advantages. Our hypothesis is that the two heads are biochemically tuned to maximize motor function such as speed, strength, processivity and microtubule affinity. To test this hypothesis, we focused on KIF3A/B, the mouse ortholog of Kinesin-2, and constructed two homodimeric KIF3 motors by fusing the head of one chain to the rod-tail of the other.

In microtubule gliding assays the two homodimeric motors moved at 10-fold different rates. Regarding the stepping rate of the wild-type KIF3A/B, the data are consistent with a coordinated head model in which detachment of the slow KIF3A head from the microtubule is accelerated roughly three-fold by the KIF3B head. However, further experiments indicated that the bulk of the difference between the two heads is due to point mutations in the A head.

For higher resolution analysis of motor function, fluorescently labeled heterodimeric and homodimeric KIF3 motors were constructed by replacing the tail domains with EGFP. The run length (processivity), dwell time, and velocity of motors
moving along immobilized microtubules were characterized by total internal reflection fluorescence microscopy (TIRF) at the single molecule level. The results showed that KIF3A/B is processive but less processive than conventional kinesin, and homodimeric KIF3A/A is slightly more processive than homodimeric KIF3B/B.

Using two dynamic fluorescence techniques, the kinetics of motor-microtubule interactions in solution were investigated. On fast time scales, rapid rotational diffusion was measured using time-resolved fluorescence anisotropy, and while on slow time scales, the translational diffusion was measured using fluorescence correlation spectroscopy (FCS). We successfully applied FCS in determining equilibrium dissociation rate constant of kinesin binding to microtubules by quantitating fractions of the rapid diffusing component (free motors) and slowly diffusing component (motor-microtubule complexes) at a range of microtubule concentrations.

The measured processivity and stepping rates were incorporated into kinetic schemes for the kinesin chemomechanical cycle, and were simulated using a Monte Carlo approach. Simulation results supported the ATP Induced Detachment (AID) model, in which the intramolecular strain and conformation change induced by the binding of ATP to the front bound head is required for detaching the rear head from the microtubule.

This work provides the most detailed characterization of Kinesin-2 motors to date, enabling insights into how these motors are optimized for their specific cellular tasks.
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Chapter 1  INTRODUCTION AND BACKGROUND

1.1 KINESIN SUPERFAMILY

Kinesins comprise a large family of molecular motors that transport intracellular cargo along microtubules using the energy derived from ATP hydrolysis. These motors consist of a well conserved ATP and microtubule binding core, a stalk region and a highly variable tail domain that is responsible for cargo interaction in diverse cellular tasks such as intracellular transport, the positioning of organelles, and movement of chromosomes during cell division.

1.2 KINESIN-2 IS DISTINCTIVE IN HAVING TWO NONIDENTICAL HEAD DOMAINS

Of the 14 classes of kinesins (Miki et al., 2001), Kinesin-2 motors are unique in that they form a heterotrimeric complex composed of two different heavy chains and a single non-motor kinesin-associated polypeptide (KAP) that binds to the motor tail. Since the work here is concerned with the motor characteristics, wild-type Kinesin-2 is generally referred here as heterodimeric.

The Kinesin-2 heterotrimeric structure is conserved between humans and Chlamydomonas, species that represent more than a billion years of evolution (Hedges, 2002), which suggests that having two non-identical heads is important for these motors to carry out their intracellular tasks. The two head domains of KIF3A/B, the mouse ortholog of Kinesin-2, share 69% sequence identity between one another. When sequences of the KIF3A and KIF3B motor domains are aligned and regions of divergence mapped to the crystal structure of conventional kinesin (Kozielski et al., 1997; Kull et al.,
1996; Vale et al., 2000), there are suggestions of functional differences surrounding the neck linker interaction region, nucleotide binding pocket, and microtubule binding region. These regions with clear sequence divergence between KIF3A and KIF3B may be important for tuning motor characteristics, but this hypothesis has not been tested.

Despite a body of both in vivo and in vitro work on Kinesin-2 structure and function, the advantage conferred by having two different heads in Kinesin-2 is not clear. This design of two different heads may be an adaptation for transport along diverse microtubule forms, or it may enable a graded response to mechanical loads or subtler forms of motor regulation.

### 1.3 INTRACELLULAR FUNCTIONS OF KINESIN-2 MOTORS

Members of the Kinesin-2 subfamily, including KIF3A/B in mouse (Yamazaki et al., 1995), KLP64D/68D in *Drosophila* (Pesavento et al., 1994; Stewart et al., 1991), FLA10 in *Chlamydomonas* (Walther et al., 1994), KRP85/95 in sea urchin (Cole et al., 1993), Xklp3A/B in *Xenopus laevis* (De Marco et al., 2001; Le Bot et al., 1998), and Osm3 in *C. elegans* (Shakir et al., 1993), have been shown to be microtubule plus end-directed motors that are involved in diverse intracellular tasks, such as anterograde intraflagellar trafficking (Cole et al., 1998; Orozco et al., 1999), assembly and maintenance of cilia and flagella (Brown et al., 1999; Cole et al., 1998; Signor et al., 1999), endoplasmic reticulum to Golgi membrane transport (Le Bot et al., 1998) and dispersion of melanosomes (Tuma et al., 1998).

KIF3A/B plays an essential role in embryonic development. KIF3A and KIF3B knockout mice displayed severe cardiac abnormalities and loss of left-right asymmetry.
This lethal defect was traced to an immotile nodal cilia that is proposed to generate a gradient of putative morphogen along the left-right axis in the node to produce a leftward flow of extraembryonic fluid (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999).

In other cells Kinesin-2 motors have also been shown to be involved in transport and development of cilia, flagella, and associated processes. In vertebrate photoreceptors, Kinesin-2 motors localize to a nonmotile cilium connecting the inner segment of the cell, where proteins are synthesized, to the outer segment, where light is sensed. Deleting these motors leads to defective protein localization and apoptosis (Beech et al., 1996; Marszalek et al., 2000; Muresan et al., 1997; Muresan et al., 1999). In *Chlamydomonas* flagella, the bi-directional transport of protein complexes along flagellar microtubules is critical for the development and maintenance of the flagella that these organisms use to propel themselves through water (Kozminski et al., 1995). This intraflagellar transport is driven by Kinesin-2 and dynein motors that move along the outer doublet microtubules that make up the flagella – disruption of the minus-ended dynein leads to accumulation of material at the end of the flagella and deletion of the Kinesin-2 ortholog FLA10 disrupts anterograde intraflagellar transport entirely and leads to flagellar retraction (Cole et al., 1998; Kozminski et al., 1995; Morris and Scholey, 1997). Because no other kinesins have been shown to transport material along axonemal microtubules, Kinesin-2 appear to be the dominant, if not the exclusive anterograde motors in cilia and flagella.

Due to these diverse cellular roles, the investigation of Kinesin-2 will provide several insights into human diseases. First, Kinesin-2 motors play an important transport
role in vertebrate retinal cells and site-specific knockout results in mislocalization of opsin and eventual apoptosis (Goldstein, 2001; Marszalek et al., 2000). Second, mice lacking one subunit of KIF3A/B showed cardiac developmental defects and a loss of left/right asymmetry, a phenotype similar to Kartagener’s syndrome in humans (Hirokawa, 2000; Hirokawa, 2000; Marszalek et al., 1999). Third, because Kinesin-2 motors have been known to play an important role in the development and maintenance of sperm flagella (Kierszenbaum, 2002; Miller et al., 1999), understanding the mechanism underlying Kinesin-2 motility may provide insights into sperm motility defects that result in infertility. Fourth, because of the importance of other kinesins in mitosis, there is an effort underway to identify kinesin inhibitors that could be used as anticancer therapeutics. Identifying mechanisms of kinesin motility may open the way for alternative strategies and target molecules for inhibiting mitotic motors.

1.4 KINESIN PROCESSIVITY

The most thoroughly studied member of the kinesin superfamily is conventional kinesin (Kinesin-1). It was the first kinesin discovered (Vale et al., 1985), and was the first cytoskeletal motor shown to be processive, defined as the ability of a single motor taking many steps along its filament track before dissociating (Howard et al., 1989). This property is important for long distance transport, especially in cases where one or only a few motors are bound to a small organelle or vesicle. To move processively, the two heads of a kinesin must coordinate such that one head is always bound to the microtubule. Coordination between motor domains has been demonstrated in previous motility and biochemical studies on kinesins, myosins and dyneins (Asbury et al., 2003;
De La Cruz et al., 2001; De La Cruz et al., 1999; Gilbert et al., 1998; Hackney, 1994; Hancock and Howard, 1998; Hirakawa et al., 2000; King and Schroer, 2000; Ma and Taylor, 1997), suggesting that interdomain coordination is ubiquitous.

1.5 COMPETING MODELS OF CHEMOMECHANICAL COORDINATION

There are two main competing structural models: the hand-over-hand model (Figure 1.1A) and the inchworm model (Figure 1.1B). The hand-over-hand model explains processive motility by a mechanism in which the two heads alternately step past each other along the microtubule (Hackney, 1994; Howard, 1996). This model is widely accepted because it easily explains three clear experimental conclusions about conventional kinesin: conventional kinesin is processive (Block et al., 1990; Hackney, 1995; Howard et al., 1989), the motor takes 8 nm steps along the microtubule following the protofilament axis (Ray et al., 1993; Svoboda et al., 1993), and the motor hydrolyzes one ATP per 8 nm step (Coy et al., 1999; Hua et al., 1997). However, the hand-over-hand model predicts that each step along the microtubule should produce an 180° rotation of the motor coiled-coil stalk, which is not supported by experimental data (Hua et al., 2002). This lack of rotation can be accounted for by the inchworm model, in which one of the two heads is always leading and undergoes an 8 nm powerstroke, the second head plays only a supporting role, and the dimeric molecule alternates between a stretched conformation (heads on successive binding sites 8 nm apart) and a contracted conformation (heads on the identical binding site or equivalent sites on adjacent protofilaments) (Hua et al., 2002). To account for the three key experimental findings, it is posited that in the inchworm model, the leading head hydrolyzes one ATP per 8 nm
step and the lagging head is enzymatically inactive. Another form of the hand-over-hand model, the asymmetric hand-over-hand model, is postulated based on the observation that the kinesin neck coiled-coil does not rotate 180° from the beginning of one step to the beginning of the next (Hua et al., 2002). In this asymmetric mechanism, the three-dimensional structures at the beginning of consecutive 8-nm steps are different and the neck linker domains of the two heads exist in two distinct conformations alternating in successive enzymatic cycles such that no rotation of coiled-coil is predicted (Hoenger et al., 2000; Hua et al., 2002).

In a recent study a “cysteine-light” kinesin, labeled with a single fluorophore located at the end of one head domain, was observed to move with alternating 17 nm and 0 nm steps instead of a uniform step size of 8 nm, strongly supporting the hand-over-hand model and ruling out the inchworm model for this motor at limiting ATP levels (Yildiz et al., 2004). The same experiments performed on myosin V also provided strong support on the hand-over-hand model for this motor (Yildiz et al., 2003). In another study, Kaseda et al. generated a heterodimeric conventional kinesin with a mutation in the ATP binding site of one head and found that the motor took alternating fast and slow steps along microtubule (Kaseda et al., 2003), providing further evidence for the hand-over-hand model. Neither of these results can be accounted for by the inchworm model.

Asbury and colleagues found that even in some truncated homodimeric kinesins the stepping rates differ between the two heads, presumably due to structural asymmetries in the coiled-coil region (Asbury et al., 2003), pointing towards the asymmetric hand-over-hand mechanism.
In both cases the motor walks along the microtubule with 8 nm steps, keeping at least one head bound to the filament at all times. For the hand-over-hand model each head takes alternating 16 nm steps, while in the inchworm model the leading head takes an 8 nm step followed rapidly by the trailing head.

To define the detailed conformational changes coupled to the kinesin ATP hydrolysis cycle, several models for kinesin’s chemomechanical cycle have been proposed based in the framework of the hand-over-hand model (Cross et al., 2000; Hancock and Howard, 1999; Rice et al., 1999; Rosenfeld et al., 2003; Schief and Howard, 2001; Schnitzer et al., 2000; Vale and Milligan, 2000). Some key features in these models that have been broadly agreed upon are as follows. 1) Kinesin is a tightly coupled motor, hydrolyzing one ATP for each 8 nm step (Coy et al., 1999; Hua et al., 1997; Iwatani et al., 1999; Schnitzer and Block, 1997). 2) Binding of ATP to the bound head precedes the attachment and ADP release by the tethered head (Hackney, 1994). 3) Attachment and detachment of the motor to the microtubule is nucleotide dependent, such that the bound head will only detach in the weak binding states, ADP or ADP • P_i (Crevel et al., 1999; Cross et al., 2000; Hancock and Howard, 1999).

Despite these consistencies, many important features of the conformational changes that occur during the ATP hydrolysis cycle are not clear. One big debate concerns the release of the rear head from the microtubule. One side proposes that the

Figure 1.1 Two competing structural models, the hand-over-hand model (A) and the inchworm model (B).
strain produced by the binding of the front head is sufficient to pull the rear head off (Cross et al., 2000; Hancock and Howard, 1999), while the other side proposes that binding of ATP to the front head is also required in addition to the intramolecular strain (Rice et al., 1999; Vale and Milligan, 2000). There are other uncertainties, such as whether phosphate releases from the attached rear head (Crevel et al., 1999; Cross et al., 2000; Hancock and Howard, 1999) or releases after the rear head detaches, and whether ATP hydrolysis occurs before the tether head attaches or after both heads bound (Crevel et al., 1999; Ma and Taylor, 1997; Rice et al., 1999).

Hence a large part of the mechanism underlying kinesin’s chemomechanical cycle has not been resolved for conventional kinesin and there are still lots of open questions. Little characterization has been done for other classes of kinesin, and difference in the mechanism may be expected due to different intracellular functions.

1.6 KINESIN-2 IS THE IDEAL CANDIDATE FOR STUDying CHEMOMECHANICAL COORDINATION

One of the difficulties of studying kinesin coordination comes from the fact that the two motor heads are identical, making it hard to distinguish the relative contribution of each head to the motility, ATP hydrolysis, and force production of the dimer. Because Kinesin-2 motors naturally have two different motor domains, they provide a powerful tool both for testing competing models of motility and for studying the coordination in dimeric motors in general. Since much supporting evidence for the hand-over-hand mechanism comes from truncated kinesins or from kinesins with one of the two heads mutated, native heterodimeric motor KIF3A/B, the mouse Kinesin-2 ortholog is a better
model of study for testing these coordination models. The approach of characterizing the properties of each KIF3 head and comparing their function to the intact KIF3A/B motor should provide important and novel insights into the nature of chemomechanical coordination in all kinesins, and to multimeric motor proteins in general. My studies are aimed to reveal the functional differences and coordination between the two heads of Kinesin-2 to understand the role the two heads play in motor function.
Chapter 2  MOTILITY OF KINESIN-2 MOTORS IN MICROTUBULE

GLIDING ASSAY

2.1 INTRODUCTION

Of the 14 known classes of kinesins (Miki et al., 2001), Kinesin-2 motors are distinctive because they contain two non-identical motor domains. Kinesin-2 motors are involved in ciliogenesis, intraflagellar transport and Golgi trafficking. KIF3A/B, the mouse Kinesin-2 ortholog, functions as a motor for anterograde axonal transport (Kondo et al., 1994; Yamazaki et al., 1995) and plays an essential role in embryonic development. Because of their unique heteromeric structure and diverse cellular roles, it is important to better understand the mechanism underlying Kinesin-2 motility.

Conventional kinesin was the first cytoskeletal motor shown to be processive, defined as the ability to take many steps along its filament track without falling off (Howard et al., 1989). Subsequently other kinesins, myosins and dyneins have also been shown to be processive transport motors (Mallik et al., 2004; Mehta et al., 1999; Okada and Hirokawa, 1999). To prevent detachment and rapid diffusion away from the microtubule, the two heads of a dimeric kinesin must coordinate such that one head is always bound to the microtubule. Because of this coordination, uncovering motor function requires not only defining the ATP hydrolysis cycle and associated conformational changes, but also identifying steps in the cycle in which the activity of one head modulates the kinetics of the second head.

Despite considerable work, there is no consensus mechanism by which conventional kinesin’s two heads coordinate their chemomechanical cycles to ensure processivity. Existing models of the kinesin walking cycle incorporate a number of
different mechanisms to ensure that the microtubule-bound head does not detach before
the tethered head binds to the next binding site. These include i) strain-dependent
detachment of the rear head (Hancock and Howard, 1998; Rice et al., 1999), ii) slowed
ATP binding to the forward head when both heads are bound (Klumpp et al., 2004;
Rosenfeld et al., 2003; Rosenfeld et al., 2002), and iii) very fast attachment and ADP
release by the tethered head (Crevel et al., 2004; Hackney, 2002). Because the strain-
dependent transitions that ensure processivity are intimately linked to the force dependent
steps, defining this coordination is crucial for understanding chemomechanical coupling
in kinesins.

Since KIF3A/B naturally has two different motor domains, it provide an
important tool for studying intersubunit coordination in dimeric motors in general. A
fundamental question concerning the coordination between the two heads of KIF3A/B is
whether it is processive or not. To be processive, the two heads must coordinate in a way
such that at least one head is always bound to the microtubule. Hence studies of
processivity will provide insights into the coordination between two heads.

In order to study the two heads of KIF3A/B individually, chimaeric homodimers,
KIF3A/A and KIF3B/B were created by fusing the head of one subunit to the rod and tail
of the other and then coexpressing with its relevant wild-type subunit in the baculovirus
expression system. In this chapter, microtubule gliding assays were used to investigate
the motility and processivity of baculovirus expressed wild-type KIF3A/B and chimaeric
homodimers.

Based on the microtubule gliding assay, there are two different methods to
determine motor processivity: microtubule pivoting assay and landing rate assay. In the
microtubule pivoting assay, motors are diluted to single molecule level so that when these low density motors adhere to glass surface one microtubule can only interact with one single motor molecule. In this case, microtubules are tethered to single motor molecules and the torsional flexibility of the motors are so high (Hunt and Howard, 1993) that we can see the diffusive rotation of microtubules, whereas at high densities, microtubules are restrained by multiple motor molecules attached along their lengths and no pivoting can be seen. Hence if the microtubule is observed to move and swivel over a single nodal point suggesting a single motor molecule is sufficient to propel microtubule movement, then this motor is processive.

However coincident co-localization of more than one nonprocessive motors at the contact point on the surface may generate the same pivoting motility. To quantitatively and statistically demonstrate motor processivity, we need to perform landing rate assays, in which the number of microtubules that land and move across unit surface area during unit time is determined at varying motor surface densities. The microtubule landing rate data are then fit to the model of Equation. 2.1 (Hancock and Howard, 1998).

\[
LR (\rho) = LR_{\text{max}} \left(1 - e^{-\rho}/\rho_0\right)^n , \tag{Eq. 2.1}
\]

where \(LR\) is the landing rate (in microtubules \(\cdot\) sec\(^{-1}\) \(\cdot\) mm\(^{-2}\)); \(\rho\) is the surface density of motors (in motors/\(\mu\)m\(^2\)); \(LR_{\text{max}}\) is the maximum landing rate (at saturating motor concentrations); \(1/\rho_0\) is the area over which a motor can interact with a microtubule; and \(n\) is the number of motors necessary to move a microtubule (equal to 1 for processive motors) (Hancock and Howard, 1998).

Based on this model, the landing rate will vary as the \(n\)th power of motor density at low motor densities. So if at low motor densities the landing rate shows a first order
dependence on motor density, then the motor is processive. For nonprocessive motors, a much higher order dependence is expected for this assay. We have performed both microtubule pivoting assay and landing rate assay on wild-type and chimaeric KIF3 motors.

2.2 MATERIALS AND METHODS

2.2.1 Expression constructs

Full-length cDNAs for KIF3A and KIF3B were a gift of L. Wordeman and L. Ginkel (University of Washington, Seattle, WA). Sequences were modified by PCR-based mutagenesis and QuikChange mutagenesis (Stratagene, Inc.) to introduce proper restriction sites and tags for purification. For KIF3A, a BglII site was added upstream of the coding sequence, the sequence coding for QKLISEEDL was appended to the final E of the coding sequence to generate a Myc tag, and an EcoRI site was added downstream of the stop codon. For KIF3B, a sequence coding for a hexa-histidine tag was introduced to the 3’ end of the KIF3B coding sequence, and a BamHI site was added following the stop codon. Two transfer vectors, pAcKIF3A and pAcKIF3B, were obtained by ligating the KIF3A and KIF3B genes into pAcUW51 baculovirus transfer vectors (Pharmingen, Inc., San Diego, CA). As initially we could only express the KIF3A subunit but not the KIF3B subunit, we compared the upstream sequence of the KIF3B gene to the consensus sequence from 154 native baculovirus genes (Ayres et al., 1994) and to other studies on baculovirus protein expression (Hirokawa and Noda, 2001; Pierce et al., 1999) (Patent US5194376). We concluded that sequences directly upstream of the ATG start codon must inhibit either transcription of the KIF3B gene or translation of the message. Hence,
the sequence AAAT was inserted immediately upstream of the start codon for KIF3B gene by site-directed mutagenesis, which enhanced expression of the KIF3B subunit.

Previous work has shown that KIF3A and KIF3B and other Kinesin-2 motors preferentially form heterodimers through their coiled-coil regions (De Marco et al., 2001; Rashid et al., 1995; Yamazaki et al., 1995), so to make homodimeric KIF3 motors containing two identical head domains (KIF3A/A and KIF3B/B), two chimaeric genes were created by switching the heads. By comparing the amino acid sequences of the KIF3A/B heads to conventional kinesin sequences from human, fly, and rat, and to the rat kinesin dimer crystal structure (Kozielski et al., 1997), we identified a ten residue identity region in KIF3A/B spanning the end of the neck linker and the start of the neck coiled-coil (Figure 2.1A). Splicing the heads in this identity region maintained the entire neck linker and head as an intact domain and the entire predicted coiled-coil as heterodimer.

To make the chimaeric KIF3 genes, we inserted a NotI site upstream of the KIF3A gene (there was an existing NotI site upstream of the KIF3B gene), and introduced silent mutations to create an AflII site at LLR in the neck-coil region of both genes (Figure 2.1A). For KIF3A, the DNA sequence CTGCTCCGC was changed to CTCTTAAGA and for KIF3B the sequence CTGCTTCGA was changed to CTCTTAAGA. The resultant pAcKIF3A and pAcKIF3B plasmids were then digested with NotI and AflII restriction enzymes (New England Biolabs, Inc., Beverly, MA), gel purified, and the heads spliced to their complementary rod-tail domains (Figure 2.1B).
Figure 2.1 (A) Amino acid sequence alignment for mouse KIF3A and KIF3B and human conventional kinesin heavy chain (HsKHC) genes at the neck-coil junction. Secondary structure predictions were taken from the rat KHC crystal structure (Kozielski et al., 1997) and the start of the coiled coil of KIF3A and KIF3B was inferred by comparison to the conventional kinesin sequence and by predictions from the COILS program. There is an obvious splice site in the neck linker region of KIF3A and KIF3B; the arrow denotes where the AflIII restriction site was introduced. (B) Constructing mutant KIF3A/A. KIF3A and KIF3B plasmids were digested and the sequence for the KIF3A head domain was spliced to the sequence for the KIF3B rod and tail domains. This chimaeric gene was then co-expressed with the wild-type KIF3A gene in insect cells, producing a mutant protein that has two KIF3A heads and the normal KIF3A/B rod and tail structure. An analogous approach was used to make KIF3B/B. GenBank accession numbers: KIF3A,
2.2.2 Protein expression and purification

Four different stocks of recombinant viruses were generated by co-transfecting KIF3 plasmids with BaculoGold™ linearized Baculovirus DNA (PharMingen, Inc.). Wild-type KIF3A/B motors were expressed by co-infecting Spodoptera frugiperda (Sf9) insect cells with wild-type KIF3A virus and KIF3B virus. Mutant KIF3A/A homodimers were expressed by co-infecting cells with chimaeric KIF3A virus and wild-type KIF3A virus; to make KIF3B/B, cells were co-infected with chimaeric KIF3B virus and wild-type KIF3B virus. Maximum yields of functional KIF3A/B, as measured by SDS-PAGE and motility assays, were achieved by growing the cells in Sf-900 II SFM serum-free medium (Gibco-BRL, Gaithersburg, MD) at 27°C, harvesting the cells 60 hours after infection and lysing the infected cells in lysis buffer with 1% Triton. For large-scale expression, 25 ml each of two recombinant viral stocks with a titer of ~1×10^8 plaque forming units (pfu)/ml were added into 500 ml of Sf9 suspension cell cultures. After 60 h incubation at 27°C, infected cells were pelleted by centrifuging for 10 min at 1000 × g, frozen in liquid nitrogen and stored at –80°C.

For protein purification, cell pellets were thawed, resuspended in lysis buffer (20 mM TrisHCl, 500 mM NaCl, 10 mM imidazole, 1 mM MgCl₂, 1% Triton, 5 mM β-mercaptoethanol, 0.5 mM MgATP, Protease Inhibitor Cocktail (PharMingen, Inc.), pH 7.5) and lysed on ice for 45 min. The crude cell lysate was then centrifuged for 30 min at 100,000 × g to remove cellular debris and insoluble proteins. His-tagged KIF3 motors
were purified by passing through a 2 ml nickel-nitrioltriacetic acid (Ni-NTA) chromatography column (QIAGEN, Inc., Valencia, CA). The column was first equilibrated with lysis buffer and then the cleared lysate was loaded onto the column, followed by 10 column volumes of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 60 mM imidazole, 1 mM MgCl₂, 10% glycerol, 5 mM β-ME, 0.1 mM MgATP, pH 7.0) to remove contaminating insect host proteins. Motor proteins were eluted from the column by a step elution with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, 1 mM MgCl₂, 10% glycerol, 5 mM dithiothreitol, 0.1 mM MgATP, pH 7.0). The protein absorbance at 280 nm was monitored during the purification process. Peak fractions were collected, frozen in liquid nitrogen and store at –80°C.

Motor concentrations were quantified by running samples on 7% SDS-PAGE gels along with BSA standards, and staining with Coomassie Blue dye. Gel images were captured by a UVP BioChem System (UVP, Inc., Upland, CA) and the optical density for each band was analyzed with LabWorks 4.0 (UVP, Inc.).

2.2.3 Hydrodynamic analysis

For sedimentation velocity analysis, 500 µl purified KIF3A/B motors were exchanged into BRB80 buffer (80 mM Pipes, 1 mM EGTA, 1 mM MgCl₂, pH 6.9) with 100 µM MgATP, layered on a 5-25% (w/v) sucrose density gradient and centrifuged at 41000 rpm for 24 hours at 4°C (Beckman L8-70M ultracentrifuge, SW 41 Ti rotor). Fractions were collected by gravity from the bottom of the gradient. Standard proteins with known sedimentation values (carbonic anhydrase, 3.2 S; BSA, 4.4 S; alcohol dehydrogenase, 7.6 S; β-amylase, 8.9 S) were run in a parallel tube. To determine the
peak fractions of the standards, Coomassie Blue stained gels were scanned and the band intensities were fit with Gaussian distributions. Motor peaks were located by motility assays. Sedimentation values of motors were then determined from the standard curves generated by a linear regression of the fraction number versus the sedimentation coefficient.

For gel filtration analysis, 100 µl KIF3A/B motors were loaded onto a Superdex 200 10/300 GL column (Amersham Biosciences, Piscataway, NJ). Due to nonspecific adsorption of motors to the gel filtration matrix, the column was run at 4°C in a high ionic strength buffer containing 50 mM sodium phosphate, 300 mM NaCl, 5 mM dithiothreitol, and 10 µM MgATP. The same standards as for the density gradients were run in parallel. Elution volumes and partition coefficients $K_{av}$ were obtained by monitoring the absorbance at 280 nm. Motor protein Stokes radius was determined from a linear regression of $(-\log K_{av})^{1/2}$ versus Stokes radius for standard proteins. The molecular weight of motor protein was then calculated using the sedimentation coefficient and Stokes radius in the Siegel and Monty equation (Siegel and Monty, 1966). In this equation, partial specific volumes for motor proteins were calculated from those volumes of the constituent amino acids using a program called SEDNTERP. For example, the partial specific volume for KIF3A/B was calculated to be 0.7300 cm$^3$·g$^{-1}$. The solvent density and solvent viscosity were chosen to be 0.99823 g·cm$^{-3}$ and 0.01002 g·cm$^{-1}$·sec$^{-1}$, respectively, which are the values for water at 20°C.
2.2.4 In vitro motility assays

Tubulin was extracted from bovine brain by repeated cycles of polymerization and depolymerization using standard recipes (Wagner et al., 1991; Williams and Lee, 1982), labeled with 5-(and 6-)-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes, Inc., Eugene, OR) (Hyman et al., 1991), and then polymerized into microtubules.

KIF3 motility was tested in microtubule gliding assays following standard procedures (Howard et al., 1993). Flow cells were first preloaded with BRB80 buffer containing 0.5 mg/ml casein to block the glass surface for 5 min, and purified motors diluted in BRB80CA (BRB80, 0.2 mg/ml casein, 1 mM MgATP) were then introduced into the chamber and allowed to adhere to the surface. After 5-10 min, motility solution (BRB80, 10 μM taxol, 1 mM MgATP, 32 nM rhodamine-labeled microtubules and an oxygen scavenger system consisting of 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase and 0.5% β-ME) was flowed into the flow cell. To obtain short microtubules with lengths of 1-5 μm, microtubules were sheared by passing the motility solution twice through a 30-gauge needle at a flow rate of 100 μl/s.

To improve the motility in the assay at low motor surface densities, an initial pre-coating step was added by introducing 10 μg/ml anti-His antibody (Novagen, Inc., Madison, WI) into the chamber.

2.2.5 Video microscopy and data analysis

Microtubule gliding was monitored by fluorescence microscopy with an upright Nikon E600 microscope (100×, 1.3 N.A. objective). Fluorescence images were captured by an intensified CCD camera (Genwac GW-902H), recorded onto S-VHS videotapes
and analyzed offline using the imaging processing software Scion Image (Scion Corporation, Frederick, MD). The distances traveled by microtubules were measured by tracing the microtubule position by hand on a transparent sheet over the video screen or by a custom tracking program. The minimum detectable threshold was 0.3 µm.

To investigate the processivity of KIF3 motors, a landing rate assay was performed at varying motor surface densities by counting the number of microtubules longer than 1 µm that landed and moved for at least 0.3 µm across motor coated-surfaces during an appropriate time window in the whole video screen area (equivalent to 3016 µm$^2$ in the flow cell). The microtubule landing rate data were then fit to a model as previously described (Hancock and Howard, 1998).

2.3 RESULTS

2.3.1 Expression and purification of recombinant KIF3A/B protein

It has been reported that KIF3 motors cannot be functionally expressed in bacteria (Kondo et al., 1994; Pierce et al., 1999), most likely due to protein aggregation and improper folding, and our work with KIF3 truncations is consistent with this (Y.C. Lee and W. Hancock, unpublished). Motivated by this, we turned to the baculovirus expression system.

After expression and purification were optimized, purified KIF3A/B appeared as a pair of bands at 85 kD and 95 kD on gels, corresponding to the KIF3A subunit and the KIF3B subunit, respectively (Figure 2.2). Sucrose density gradient centrifugation of these motors resulted in a single peak with a sedimentation value of 6.8 ± 0.1, consistent with previous data for sea urchin KRP85/95-GFP dimer (6.3 ± 0.4) (Pierce et al., 1999).
When analyzed by gel filtration, there was a motor peak with a calculated Stokes radius of 5.4 nm and predicted molecular mass 152.5 kD. This agrees well with the predicted 167.7 kD for the KIF3A/B heterodimer, showing that our recombinant KIF3A/B is indeed heterodimeric.

![Figure 2.2](image.png)

Figure 2.2 SDS-PAGE of purified wild-type KIF3A/B heterodimer. Lane 1, molecular weight markers; lane 2, eluate of KIF3A/B. This figure is a reprint from the published article by Zhang and Hancock (Zhang and Hancock, 2004).

From gel densitometry, some KIF3A/B preps showed a 1:1 stoichiometry of KIF3A subunits to KIF3B subunits, but in other preps the stoichiometry of KIF3B to KIF3A ranged from 2:1 to 7:1. Although there was no observable difference in motility between these preps and the 1:1 stoichiometry preps, we wanted to characterize the oligomerization state of this KIF3B and rule out the possibility that any excess KIF3B was affecting our motility assays. From the gel filtration analysis there was no evidence of KIF3B monomer peak at the predicted 86.3 kD MW, but there was a large protein peak that eluted after one column volume, which we interpreted as nonspecific adsorption of motors to the column (as seen by others (Pierce et al., 1999)), and there was a protein peak that ran with the void volume (MW > 600 kD), consistent with higher order oligomers of KIF3B. To test whether KIF3B alone is functional, we infected cells with
only the KIF3B virus and purified and tested the resultant protein. In these KIF3B preps there was not an additional ~70 kD band corresponding to the native KIF3A ortholog from the insect cells, indicating that KIF3B does not heterodimerize measurably with native Sf9 proteins. From gel filtration analysis, there was no evidence for either KIF3B monomers or dimers, but again there was protein both in the void volume and in a late fraction, suggesting that this KIF3B formed aggregates and/or was partially denatured and interacted nonspecifically with the column. When tested in motility assays, this purified KIF3B showed only minimal microtubule binding (8-fold lower than KIF3A/B at comparable motor concentrations), and no microtubule movement was observed, confirming that they are not functional motors. Finally, to test for possible effects on KIF3A/B motility, we added a 7-fold excess of this purified KIF3B to purified KIF3A/B in motility assays and found no effect on the landing rate and an only minimal effect on the microtubule gliding speed (when 350 nM KIF3B was mixed with 50 nM KIF3A/B, the microtubule gliding speed decreased from 164 ± 36 nm/s to 145 ± 24 nm/s (mean ± SD)). These results led us to conclude that any extra KIF3B in our motor preps is denatured or partially unfolded protein that has no effect on KIF3A/B motility.

2.3.2 KIF3A/B is a processive motor optimized for long-distance transport

To investigate whether KIF3A/B is processive, the motor activity of KIF3A/B was measured at a series of motor surface densities in the microtubule gliding assay. The surface density of attached KIF3A/B motors was varied by loading different concentrations of motors into the flow cell. Assuming that all molecules loaded are absorbed onto the surface and half of them land on each face of the flow cell, the motor
surface density is estimated by the product of the protein molar concentration and the flow cell volume divided by the area of both flow cell surfaces. Hence, for our stock of purified KIF3A/B with concentration 110 nM estimated by gel scanning, the maximum surface density was calculated to be 3900 molecules/µm² based on the flow cell dimension of 18 mm × 7 mm × 119 µm.

2.3.2.1 Velocity of microtubule movement is independent of KIF3A/B surface density

Microtubule gliding velocity was assessed at a variety of KIF3A/B surface densities from 19.5 molecules/µm² to 3900 molecules/µm². As seen in Figure 2.3A, the gliding speed is invariant over several decades of motor density. Even when the motor surface density was decreased to single molecule levels (19.5 molecules/µm²), KIF3A/B was capable of propelling microtubules at the same velocity as at high surface densities. The average velocity was 184 ± 28 nm/s (mean across all densities ± standard deviation, n = 85).

This density independence is similar to the behavior of processive conventional kinesin and myosin V (Howard et al., 1989; Rock et al., 2000), and in contrast to the behavior of nonprocessive myosin II, which exhibits a significant drop in velocity as the motor density is decreased (Uyeda et al., 1991).

2.3.2.2 Pivoting movements of microtubules are observed at low KIF3A/B surface densities

At low KIF3A/B surface densities (3.9 – 39 molecules/µm²), microtubules were observed to swivel over single nodal points. The velocity of microtubule pivoting was estimated by measuring how fast the leading end of a microtubule moved away from the nodal point. Pivoting microtubules moved relative to the contact point with the same
speed as non-pivoting microtubules at high KIF3A/B densities, indicating a KIF3A/B molecule was located at the nodal point and not some low level contaminant.

An example of microtubule pivoting movement is shown in Figure 2.3B. The microtubule lands on the surface, presumably tethered to one KIF3A/B molecule, pivots and moves its entire length (3.6 μm) through the single nodal point, then detaches and diffuses away. Assuming 8 nm per step, the single KIF3A/B molecule under this swiveling microtubule took 450 steps until the end of the microtubule was reached. This microtubule pivoting result strongly suggests that KIF3A/B is processive.

2.3.2.3 One single KIF3A/B motor is sufficient to drive microtubule movement

To quantitatively and statistically investigate the processivity of KIF3A/B, landing rate assays were performed to determine the number of motors required to move a microtubule (Figure 2.3C). Based on the model described by Hancock and Howard (1998), at low motor densities the landing rate will vary as the $n$th power of motor density, where $n$ is the number of motors necessary to move a microtubule and appears as the slope of a log-log plot (landing rate versus motor density).

As the surface density of KIF3A/B was decreased, the fall in the microtubule landing rate was proportional to the motor density. The landing rate data were best fit with $n = 1$, suggesting that one molecule of KIF3A/B is sufficient for motility. This assay provides statistical evidence that a single KIF3A/B molecule, not an accidental co-localization of more than one nonprocessive motors, is sufficient to move a distance greater than 300 nm.
Figure 2.3 (A) Microtubule gliding speeds for wild-type KIF3A/B plotted over a range of motor surface densities. Error bars correspond to standard error of the means of at least 7 velocity determinations for each density. (B) Microtubule pivoting around a single point on the surface coated with a very low density of KIF3A/B motor. (C) Microtubule landing rate of wild-type KIF3A/B plotted as a function of motor density. Error bars correspond to standard error of the means of the landing rate from at least 5 different windows for each density. The data are best fit with $n = 1$ (solid line), indicating a single KIF3A/B molecule is sufficient to drive the movement of a microtubule. For comparison, the fit for $n = 2$ (dashed line)
To understand the coordination between the two different heads of KIF3A/B, we constructed and expressed two types of chimaeric motors, KIF3A/A and KIF3B/B, that retain the wild-type coiled-coil dimerization domain but contain two identical head domains. Both KIF3A/A and KIF3B/B could be functionally produced by the same expression and purification system as wild-type KIF3A/B.

2.3.3.1 KIF3A/A chimaera moves slowly in the microtubule gliding assay

Homodimeric KIF3A/A was capable of inducing microtubule gliding only when adsorbed at medium surface densities of roughly 400 molecules/µm². At high motor densities, numerous microtubules attached to the surface but no movement was observed. At low densities, no microtubules bound at all. At motor densities where motility could be observed, microtubules that landed on the surface moved at an average speed of 42 ± 11 nm/s (mean ± SD, n = 22).

To confirm this motor density dependent motility, we tested KIF3A/A from four different preps. The same reliable gliding speed was detected at medium KIF3A/A surface densities for all cases. To ensure that the low velocity is indeed an inherent quality of KIF3A/A rather than a biased result due to improper splicing at the neck linker region in the chimaera, we co-expressed the 3A chimaeric gene (3A head / 3B rod-tail) with the 3B chimaeric gene (3B head / 3A rod-tail) to create a heterodimer with one chain having a 3A head and a 3B rod-tail and the other having a 3B head and a 3A rod-tail. If
the splice site is appropriate, we should expect the new heterodimer to have the same velocity as wild-type KIF3A/B.

This chimaeric heterodimer moved microtubules at 169 ± 32 nm/s (mean ± SD, n = 58), consistent with the velocity of microtubules driven by wild-type KIF3A/B 184 ± 28 nm/s (n = 85). In addition, motility was observed across a range of surface densities and the velocity was independent of density (data not shown). Hence, switching heads between the two subunits at the position of our splice site doesn’t affect the motility of both homodimeric chimaeras, and slow motility of KIF3A/A is not an artifact of the splice site.

2.3.3.2 KIF3B/B chimaera is faster but less processive than wild-type KIF3A/B

KIF3B/B chimaera exhibited very robust motility across a broad range of motor surface densities. The velocity of microtubule movement driven by KIF3B/B remained constant at 446 ± 34 nm/s (mean across all densities ± SD, n = 135) through the entire range of motor densities from 15 molecules/µm² to 1500 molecules/µm² (Figure 2.4A).

At low motor densities, most microtubules moved in a straight trajectory suggesting movement by multiple motors, but a few microtubules pivoted with small angles, moved very short distances less than 1 µm, and then diffused away before the trailing ends passed the contact points. Compared to the wide-angle, long-distance pivoting behavior of single KIF3A/B motors, KIF3B/B chimaeras may have much shorter processive run lengths than KIF3A/B heterodimer. The lower processivity of KIF3B/B than KIF3A/B implies that although 3B head is capable of generating forward movement faster than the 3A head, coordination with the 3A head is required for optimal processive movement.
Landing rate assays were performed to quantitatively determine the processivity of KIF3B/B. The best fit of the data was $n = 2$ suggesting the number of KIF3B/B molecules required for motility is at least two (Figure 2.4B). Therefore, KIF3B/B is not processive at the detection level of this assay (300 nm), but we cannot rule out the possibility that KIF3B/B is processive with run lengths less than 300 nm.

**Figure 2.4** Dependence of KIF3B/B motility on motor surface density. (A) Microtubule gliding speeds for chimaeric KIF3B/B plotted over a wide range of motor surface densities, error bars correspond to standard error of the means of at least 10 velocity determinations for each density. (B) Microtubule landing rate of chimaeric KIF3B/B plotted as a function of motor density, error bars correspond to standard error of the
means of landing rate from at least 4 different windows for each density. This figure is a reprint from the published article by Zhang and Hancock (Zhang and Hancock, 2004).

2.3.3.3 The two heads of KIF3A/B have different motility properties

The two homodimers, KIF3A/A and KIF3B/B, propel microtubules at ten-fold different velocities in the microtubule gliding assay (Figure 2.5). KIF3A/A moves at 42 ± 11 nm/s, which is about five-fold slower than wild-type KIF3A/B speed of 188 ± 38 nm/s, while KIF3B/B moves at 409 ± 47 nm/s, roughly twice the speed of wild-type KIF3A/B. These results provide the first evidence that the two heads of KIF3 are functionally distinct.

**Figure 2.5** Microtubule gliding speeds for wild-type KIF3A/B and chimaeric KIF3 motors. For each motor type, the column bar represents the average of velocities determined at a range of motor densities from at least two protein preparations. Error bars correspond to the standard deviation. This figure is a reprint from the published article by Zhang and Hancock (Zhang and Hancock, 2004).

We have constructed three analytical models to interpret these velocity data (Figure 2.6). In the Independent Head Model, the cycle rates of each head in the heterodimer are identical to those in the respective homodimers, and there is no
correlation between the cycles of the two heads. From this model, which would best describe a nonprocessive motor, the predicted velocity of the heterodimer is an average of the speeds of the two homodimers. Although the data quantitatively agree with the model predictions, we exclude this model based on KIF3A/B is processivity: since the heads remain together as the dimeric motor walks along the microtubule for hundreds of steps, they can’t be moving at different speeds.

The Sequential Head Model is a simple hand-over-hand model in which the heads step sequentially along the microtubule and the cycle times for each head match those observed in the homodimers. Hence, the time it takes the heterodimer to take two steps is equal to the time it takes head A to step plus the time it takes head B to step, and the predicted velocity of the heterodimer is:

\[ V_{\text{Dimer}} = \frac{2V_A V_B}{V_A + V_B} \]

The important result is that the predicted heterodimer speed of 76 nm/s for the Sequential Head Model is significantly less than the measured KIF3A/B speed of 188 nm/s, excluding this model.

In the Coordinated Head Model the kinetic cycle of each head is modulated by the activity of the second head beyond simply waiting for the second head to complete its hydrolysis cycle. Hence, by pairing the slow A head with a fast B head in the heterodimer, the stepping rate of the A head must be faster than when it is paired with another A head in the homodimer. This can be interpreted quantitatively as follows. If all three motors take 8 nm steps and we assume that in the homodimers the kinetics of the two heads are identical, then in the homodimers each KIF3A head takes 190 msec to take a step (= 8 nm/step ÷ 42 nm/s) and each KIF3B head takes 19 msec to take a step (= 8
nm/step ÷ 409 nm/s). To account for the 85 msec needed for the KIF3A/B motor to take two successive steps (= 16 nm ÷ 188 nm/s), then the cycle of the A head must be sped up from 190 msec in the homodimer to 66 msec in the heterodimer (assuming the kinetics of the B head are unchanged). Hence, the Coordinated Head Model fits if the KIF3B head accelerates the stepping rate of the KIF3A head by a factor of 2.9.

Figure 2.6 Interpreting heterodimer velocity data. Rates are given as stepping rates (\(k\)) or stepping times (\(\tau = 1/k\)). The independent head model assumes no coordination. In the sequential model the heads alternately step along the microtubule with identical rates as in the homodimers. In the coordinated head model, the heads alternately step along the microtubule, but the rates are different in the context of the heterodimer than in the homodimers. The data can be explained if the fast head B accelerates the slow head A by a factor of 2.9 in the heterodimer. We hypothesize that this is due to accelerated detachment from the microtubule. This figure is a reprint from the published article by Zhang and Hancock (Zhang and Hancock, 2004).

### 2.4 DISCUSSION

In eukaryotic cells Kinesin-2 motors carry membranous vesicles and proteins along cytoplasmic microtubules and transport proteinaceous rafts along axonemal
microtubules. We are seeking to understand how these kinesins are optimized for their cellular tasks and what role the two different heads play in Kinesin-2 motility. Because intersubunit coordination is central to the mechanism of many homodimeric kinesins and myosins, having two non-identical heads opens a range of novel coordination mechanisms, and provides a model with which to better understand intersubunit coordination across all molecular motors.

Processivity, the ability to take many steps along the filament track without detaching, is an important property for transport motors, but compared to the body of work on conventional kinesin there is relatively little data on the processivity of the Kinesin-2 subfamily. Here, we find for the first time that a member of the Kinesin-2 subfamily is a processive motor, consistent with its role in intracellular transport. This finding for mouse KIF3A/B contrasts with work from Pierce et al. (1999) who failed to measure processive runs of KRP85/95, the sea urchin Kinesin-2 ortholog, using a single-molecule fluorescence-based assay. It is possible that this is simply due to species differences; for example, when assayed under identical conditions chick myosin-Va (M5a) was found to be processive while two yeast class V myosins, Myo2p and Myo4p, were reported to be nonprocessive motors (Reck-Peterson et al., 2001). However, a more plausible explanation for the lack of processivity of Pierce et al. is that the full-length KRP85/95 in solution is inhibited by its tail domain in the absence of cargo binding, similar to conventional kinesin (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000). In our gliding assay experiments the KIF3A/B tail is bound to the glass surface, presumably disinhibiting the motor. Our finding of KIF3A/B processivity
supports the notion that intraflagellar transport driven by Kinesin-2 motors is analogous to axonal transport driven by conventional kinesin in neurons.

Why does KIF3A/B have two non-identical heads? The Kinesin-2 heterotrimeric structure is conserved between humans and \textit{Chlamydomonas}, species that diverged more than a billion years ago (Hedges, 2002), which suggests that having two non-identical heads is important for these motors to carry out their intracellular tasks. However, despite a body of both \textit{in vivo} and \textit{in vitro} work on Kinesin-2 structure and function, this question remains unanswered. To understand what role the two KIF3A/B heads play in motor function, we have constructed two homodimeric chimaeras with identical head domains dimerized via the wild-type coiled-coil domain. The striking difference in velocity between the KIF3A/A and KIF3B/B chimaeras indicates that the two heads are functionally distinct and raises the intriguing possibility that their chemical kinetics are tuned to complement one another during processive motility.

An important consideration in designing the KIF3A/A and KIF3B/B chimaeras was where to put the splice site. Ideally, the splice site should be located just after the core motor domain and just before the coiled-coil domain that determines heterodimerization, but this is complicated somewhat by the lack of crystal structure for dimeric KIF3A/B. Fortunately, the sequences align reasonably well with conventional kinesin and, based on the crystal structure of dimeric kinesin, there is a stretch of ten conserved residues in KIF3A and KIF3B that span the end of the neck linker and start of the coiled-coil (Figure 2.1A). This is where the splice was made for our chimaeras. The fact that the double chimaera (3A head/3B rod-tail with 3B head/3A rod-tail) has similar motility to wild-type KIF3A/B indicates that the splicing itself does not measurably alter...
the motor function. Our differential head speeds contrast with an early study on KIF3A/B performed before the crystal structure of the conventional kinesin head was solved. Yamazaki et al. (1995) made two different KIF3B/B chimaeras: when the splice site was positioned in the coiled-coil dimerization region (3B head 1-359 / 3A tail 365-701), the motors were non-functional, and when the splice was positioned in the core motor domain (3B head 1-308 / 3A tail 314-701), the chimaera moved at the same speed as their reported wild-type speed of ~0.3 μm/s. For the former chimaera, the most reasonable explanation for the lack of motility is that dimerization is disrupted. For the latter chimaera, it is not surprising that it moves because the splice site is in loop 13 between α5 and β8 in the core of the motor, leaving the neck linker and dimerization domains intact. Taken together, results from the Yamazaki chimaera and our KIF3B/B chimaera suggest that residues responsible for the velocity differences between the two heads are contained in the region 309-346 of KIF3B and 314-351 of KIF3A.

What do the gliding velocities of the homodimeric constructs tell us about coordination between the two heads of wild-type KIF3A/B? If the two heads alternately step along the microtubule with identical rates as in the homodimeric motors (Figure 2.6, Sequential Head Model), the predicted heterodimer speed is dominated by the slow head, and is considerably slower than our measured rate. Hence, the data are best explained by a coordinated hand-over-hand model in which the stepping rates in the context of the heterodimer are different than the rates observed in the homodimeric motors. At a minimum, if the two heads alternately step along the microtubule then the KIF3A head must be stepping 2.9-fold faster in the context of the KIF3A/B heterodimer than in the homodimer.
What are potential coordination mechanisms that can account for this acceleration? The best paradigm in which to interpret these KIF3 results is the hydrolysis cycle for conventional kinesin, where interdomain coordination has been shown to be crucial for maintaining kinesin processivity. The problem is there is no consensus as to precisely which transitions in the cycle involve coordination. In one model of the walking cycle, it is proposed that when both heads are bound to the microtubule, forward strain produced by the leading head accelerates detachment of the trailing head (Hancock and Howard, 1998; Hancock and Howard, 1999). Processivity is maintained by ensuring that the rear head will not detach until the leading head binds. However, while this model provides a nice framework for interpreting the KIF3 data, there is debate regarding the degree to which attachment of the leading head does in fact accelerate detachment of the trailing head. Using fluorescent reporters that monitor head detachment, Rosenfeld and colleagues concluded that the acceleration of detachment by the leading head is at most a factor of 2-3 fold in a cysteine-modified human conventional kinesin construct (Rosenfeld et al., 2003; Rosenfeld et al., 2002). Using “roadblocks” on microtubules that prevent the attachment of kinesin’s leading head, Crevel et al. (Crevel et al., 2004) similarly concluded that the leading head accelerates the detachment of the trailing head by at most a factor of two in rat conventional kinesin. What does this mean for KIF3A/B? As discussed in Results, the KIF3 velocity data can be accounted for by a heterodimer model in which the fast B head speeds up the walking cycle of the slow A head by a factor of 2.9. Hence, if we assume that rear head detachment is the rate limiting step in the walking cycle, then a model in which the fast KIF3B head accelerates detachment of the slow KIF3A head in the context of the heterodimer is in reasonable
agreement with the 2-3 fold acceleration of detachment measured in conventional kinesin.

There are other coordination models that also explain the processivity of conventional kinesin. Rosenfeld and colleagues have proposed that when both heads are bound to the microtubule, rearward strain on the leading head slows ATP binding to that head until the rear head detaches and relieves this strain (Rosenfeld et al., 2003; Rosenfeld et al., 2002). This mechanism also satisfies the constraint that the rear head detaches before the forward head, ensuring that the motor takes many steps during each encounter with a microtubule. For the KIF3B head to accelerate the stepping rate of the KIF3A head, it must accelerate the rate limiting step. In this model the rate limiting step is most likely detachment of the rear head from the microtubule or a step immediately preceding it (so that the motor waits with both heads bound, the rear head detaches, and then the leading head binds ATP). Hence, the KIF3 data is again best explained by a mechanism in which the KIF3B head accelerates detachment of the KIF3A head in the heterodimer.

There are two recent studies on conventional kinesin that are relevant to understanding the kinetics of the KIF3 walking cycle. Kaseda et al. (Kaseda et al., 2003) generated a heterodimeric conventional kinesin with a mutation the ATP binding site in one head and found that the motor took alternate fast and slow steps along microtubule. Interestingly, the step duration in a homodimer consisting of two mutant heads matched the step duration of the slow head in the heterodimer, showing that in this mutant the fast head does not affect the kinetics of the slow head (our Sequential Head Model, Figure 2.6). In another study, Asbury and colleagues (Asbury et al., 2003) found that even in
some homodimeric kinesins the stepping rates differ between the two heads, presumably due to structural asymmetries in the coiled-coil region. These and other findings point towards an asymmetric hand-over-hand mechanism for conventional kinesin in which the two heads, due to either structural or kinetic asymmetries, undergo distinct structural or kinetic transitions as they step along the microtubule.

If the two heads of KIF3A/B are biochemically tuned to optimize the performance of the intact heterodimer, then we expect there to be other differences beyond simply the unloaded stepping rate. For instance, if the slow head is responsible for maintaining association with the microtubule, then we would expect the slow homodimer to have a greater microtubule affinity than the fast homodimer. Alternatively, the two heads may be tuned such that the fast head (fast but weak) dominates under the unloaded conditions of our microtubule gliding assay, while the slow head (slow but strong) dominates at high loads. These possibilities are currently being tested using single molecule mechanical techniques to measure the stepping rates and strength of each head.

It is possible that the design of two non-identical heads plays other roles in motor function. One possibility is that the two heads enable subtle regulation during bidirectional transport either by providing multiple sites of regulation or by enabling different cell signaling pathways to converge on the motor. A second possibility is that the two different heads provide the motor with an enhanced ability to move along axonemal microtubules; no other kinesins outside of the Kinesin-2 subfamily have been shown to transport cargo along axonemal microtubules (Cole, 1999). Though speculative, perhaps these heterodimeric motors walk along the seam of the doublet microtubules or interact optimally with the microtubule associated proteins found on
axonemal microtubules. We now know that the heterodimeric KIF3A/B is processive and that its two heads are functionally distinct. Further studies should uncover both the nature of the intersubunit coordination, and the functional advantage conferred by having two non-identical motor domains.
Chapter 3  PROCESSIVITY OF EGFP-TAGGED KINESIN-2 QUANTIFIED IN SINGLE MOLECULE FLUORESCENCE ASSAY

3.1 INTRODUCTION

In chapter 2, we used two different methods, the microtubule pivoting assay and the landing rate assay, to determine the processivity of wild-type KIF3A/B and chimaeric KIF3B/B. Because of the lack of motility at low motor surface densities, we couldn’t perform these assays to estimate the processivity of chimaeric KIF3A/A. Both of these two methods, which are based on the microtubule gliding assay, have their limitations. Although statistically improbable, the microtubule pivoting assay can be biased by co-localization of more than one nonprocessive motor at the nodal point. For the landing rate assay, it is difficult to obtain low-density motility because not all motors can retain activity when absorbed to glass surfaces. This problem is especially relevant for classes outside of Kinesin-1. In addition, in this assay it is hard to quantitate run lengths due to rebinding of microtubules to the motors on the surface. Hence, demonstrating single-molecule motility at low motor densities using microtubule gliding assays requires substantial amounts of both luck and hard work. It’s necessary to employ an alternative method to quantitatively characterize the processivity of KIF3 motors.

In this chapter, we used a single-molecule technique – total internal reflection fluorescence (TIRF) microscopy to quantify the processivity of GFP-tagged KIF3 motors and determine whether the two heads have different degree of processivity.

An increasing number of important biological investigations have been conducted using TIRF microscopy since 1981, when this technique was first applied in the study of
cell-substrate adhesions (Axelrod, 1981). TIRF microscopy, also called evanescent wave microscopy, is the best technique for measuring motor-microtubule interactions at the single-molecule level (Axelrod, 2001; Funatsu et al., 1997; Inoue et al., 2001; Okada and Hirokawa, 1999; Pierce et al., 1999; Vale et al., 1996). It does not require adsorbing motors to surfaces, it isn’t complicated by the slow diffusion of microtubules in the gliding assay, and it is rather insensitive to the presence of inactive motors in the preparation. With current optics and cameras it is reasonably straightforward to implement on a standard inverted microscope. In this technique, an excitation laser beam is directed into a solid of high index of refraction (e.g. glass) at an angle greater than the critical angle, such that when the beam encounters the interface with a liquid medium of lower refractive index (e.g. water), the light is totally internally reflected back into the solid rather than refracted through the interface. Some of the incident energy penetrates through the interface and propagates parallel to the surface in the plane of incidence. This sets up an evanescent electromagnetic field in the liquid, which exponentially decays in intensity with increasing distance normal to the surface and is capable of exciting fluorescent molecules near the surface. The space constant of the decay is described by Equation 3.1 (Axelrod, 2001).

\[
d = \frac{\lambda_0}{4\pi} \left( n_2^2 \sin^2 \theta - n_1^2 \right)^{\frac{1}{2}}
\]  

(Equation. 3.1)

where \( \lambda_0 \) is the wavelength of the light, \( n_2 \) and \( n_1 \) are the indices of refraction of the solid and the liquid, respectively, and \( \theta \) is the incident angle of the light. Typically, space constants of less than 100 nm can be obtained. Hence, this technique can restrict the excitation and detection of fluorophores to a thin region very near the surface (within \( \leq 100 \) nm) and correspondingly reduce background due to out-of-focus fluorescence in the
sample, dramatically improving the signal-to-noise ratio, and consequently the spatial resolution.

The standard geometry for TIRF illumination of the sample is to introduce a laser beam into the microscope coverslip at an angle equal to or greater than the critical angle \( \theta \geq \theta_c \) to excite fluorophores only near the surface, and to visualize the emitted fluorescence using a microscope objective and a CCD camera (Axelrod, 2001).

There are two different configurations to introduce a laser beam into the microscope coverslip at an angle \( \theta \geq \theta_c \): prism-based TIRF (Figure 3.1A) and objective-based TIRF (Figure 3.1B). In the prism-based technique, the laser is angled so that the emitted light enters a prism attached to the glass coverslip and creates an evanescent wave on the glass-specimen interface when the incident angle is adjusted to the critical angle. The fluorescence emission is collected on the opposite side of the coverslip by a microscope objective. In contrast, in the objective-based technique an objective with very high numerical aperture is employed to introduce the laser beam into the coverslip-specimen interface. When the laser beam is focused on the outer edge of the objective’s back focal plane, light can exit the objective at an angle equal to or greater than the critical value. The farther the beam focus is positioned off-axis, the larger the angle that the beam will emerge from the objective. Prism-based TIRF has several limitations: the prism restricts access to the specimen due to geometric constraints, and the illumination is introduced on the specimen side opposite to the objective, requiring imaging of the evanescent field region through the bulk of the specimen. Hence, in our study, we utilized objective-based TIRF.
To be visualized by TIRF microscopy, kinesins need to be fluorescently labeled either by chemical labeling on cysteine residues with organic dyes such as Cy3, or by generation of green fluorescent protein (GFP) fusion proteins.

We made GFP fusions instead of cysteine-containing constructs for Cy3 labeling because: i) site-specific chemical labeling involves making mutations to introduce specific cysteine residues, which could potentially inactive the motor; ii) chemical labeling requires the availability of high concentration of purified protein; iii) chemical labeling generates a mixture of proteins with different numbers of fluorophores, complicating interpretation of fluorescence data. In contrast, GFP-tagged motors are inherently fluorescent and can be used immediately after column purification.

Under the TIRF microscopy, the signal from individual green fluorescent proteins (GFP) can be detected at near video rate. Thus, fusion proteins consisting of a functional motor attached to a GFP domain can be observed interacting with surface adsorbed microtubules that are labeled with a different fluorescent dye. Using this approach, the processive movements of wild-type and mutant forms of conventional kinesin and KIF1A have been observed at single molecule level (Funatsu et al., 1997; Inoue et al., 2001;
Okada and Hirokawa, 1999; Okada and Hirokawa, 2000; Pierce et al., 1999; Vale et al., 1996).

3.2 MATERIALS AND METHODS

3.2.1 DNA construct generation for EGFP-tagged motors

To visualize single motor molecules, enhanced green fluorescent protein (EGFP) was fused to KIF3 motors. Because these constructs are to be studied free in solution, we deleted the tail domains of wild-type and chimaeric KIF3A and KIF3B to remove any tail inhibition effects (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000), and replaced them with EGFP, maintaining the C-terminal His and Myc tags for purification.

3.2.1.1 pAcKIF3A-GFP and pAcKIF3B-GFP

The plasmid containing EGFP, hEB1_pEGFP-N1, was obtained from Ram Dixit (Cyr Lab, The Pennsylvania State University, University Park, PA). A XhoI site was inserted directly downstream of the last coding amino acid of EGFP by QuikChange mutagenesis (Stratagene, Inc.), replacing the TAA stop codon with CTCG. An AvrII site was introduced to the upstream of EGFP gene by digesting the plasmid with SacII and BamHI restriction enzymes (New England Biolabs, Inc.), gel extracting the large segment with SacII and BamHI overhangs and then ligating with a two-oligonucleotide adapter containing the same SacII and BamHI overhangs and an internal AvrII site, 5’-GGCGTGTCGCCTAGGG-3’ and 5’-GATCCCCTAGGCGACACGCGCCGC-3’.

In choosing the truncation site on the KIF3A/B motor, it would be ideal to delete as much of the tail and rod as possible and retain only the dimeric heads, but dimerization
experiments on other Kinesin-2 motors indicate that the entire coiled-coil is necessary for proper heterodimerization (De Marco et al., 2001; Rashid et al., 1995; Yamazaki et al., 1995). Hence, the truncation site was based on sequence predictions of where the coiled-coil rod domain ends and the globular tail domain begins. From sequence inspection, there is a clear end to the coiled-coil at Pro\textsubscript{599} for KIF3A and Pro\textsubscript{592} for KIF3B (prolines disrupt alpha helices), and there is good sequence match between KIF3A (LIIDNFIP) and KIF3B (LIIENFIP) up to this Proline, supporting the idea that this transition to the globular tail is conserved between the two chains. Therefore, we inserted an AvrII site right after the coding sequence for amino acid Tyr\textsubscript{602} for KIF3A and Ser\textsubscript{603} for KIF3B by QuikChange mutagenesis (Stratagene, Inc.). For KIF3A, the DNA sequence CAGGAA after Tyr\textsubscript{602} was changed to CTAGGAA and for KIF3B the sequence TTTTT after Ser\textsubscript{603} was changed to CTagGG. There was an existing XhoI site directly upstream of the KIF3A Myc tag and KIF3B His tag.

The resultant pEGFP-N1 plasmid and the pAcKIF3A and pAcKIF3B plasmids were digested with AvrII and XhoI restriction enzymes (New England Biolabs, Inc.) and gel purified. Then the EGFP segment was fused into the KIF3A and KIF3B right after the coil-coil domain by ligation using T4 DNA ligase (Promega, Inc., Madison, WI).

3.2.1.2 pAcKIF3Achimaera-GFP and pAcKIF3Bchimaera-GFP

To make EGFP-tagged homodimeric KIF3 motors containing two identical head domains (KIF3A/A-GFP and KIF3B/B-GFP), two chimaeric genes (pAcKIF3AChimaera-GFP and pAcKIF3Bchimaera-GFP) were created by switching the heads following the same method as in section 2.2.1. In brief, the pAcKIF3A-GFP and pAcKIF3B-GFP
plasmids were digested with NotI and AflII restriction enzymes (New England Biolabs, Inc.), gel purified, and the heads spliced to their complementary rod-tail domains.

3.2.1.3 KHCA\text{tail-GFP}

An analogous approach was used to create EGFP-tagged conventional kinesin as a control for single molecule fluorescence measurements. The conventional kinesin we used here is called KHCA\text{tail}, which consists of two \textit{Drosophila} conventional kinesin heavy chains truncated after residue Met$_{559}$ (GenBank accession number AF161077) (Coy et al., 1999).

3.2.1.4 Point mutation reparation

The bad motility observed for EGFP-tagged KIF3 motors drove me to sequence all the gene constructs for KIF3A and KIF3B. In pAcKIF3A, there were seven point mutations and two of them were silent, including i) ACT (Thr$_{102}$) to ACC (Thr$_{102}$), ii) GGG (Gly$_{103}$) to GGA (Gly$_{103}$), iii) GGG (Gly$_{121}$) to GAG (Glu$_{121}$), iv) GCG (Ala$_{210}$) to GTG (Val$_{210}$), v) CCG (Pro$_{296}$) to TCG (Ser$_{296}$), vi) AAG (Lys$_{404}$) to AGG (Arg$_{404}$), and vii) Missing G right after Ser$_{708}$ in the Myc tag. In pAcKIF3B, there were four point mutations and two of them were silent, including i) AAT (Asn$_{20}$) to AAC (Asn$_{20}$), ii) GCG (Ala$_{34}$) to GTG (Val$_{34}$), iii) TTT (Phe$_{123}$) to TTC (Phe$_{123}$), and iv) GCC (Ala$_{465}$) to GTC (Val$_{465}$). In addition to the seven point mutations in pAcKIF3A, pAcKIF3A-GFP contained another mutation: missing AAAT right upstream of the ATG start codon of KIF3A. pAcKIF3B-GFP contains the same four point mutations as in pAcKIF3B. All nonsilent mutations were fixed by QuikChange Multi Site-Directed Mutagenesis (Stratagene, Inc.) and silent ones were ignored.
3.2.2 Protein expression and purification

KIF3A/B-GFP, KIF3A/A-GFP and KIF3B/B-GFP fusion proteins were expressed and purified in the same baculovirus expression system as described in 2.2.2. KHCΔtail-GFP was expressed in bacteria and purified by Ni column chromatography by one of our lab members, Maruti Uppalapati, following standard procedures (Hancock and Howard, 1998).

3.2.3 Multiple motor motility assays

Motility of EGFP-tagged motors was tested in microtubule gliding assays. Tubulin was labeled with Alexa Fluor® 647 carboxylic acid, succinimidyl ester, (Molecular Probes, Inc.) and then polymerized into microtubules. Alexa Fluor® 647 was chosen for minimal spectral overlap with EGFP. Since the tails of EGFP-tagged motors were deleted, 10 µg/ml anti-GFP antibodies (Vector Laboratories, Inc., Burlingame, CA) were first loaded into the flow cell to help absorption of motors onto the surface. After 5 min, unbound antibodies were washed away and BRB80 buffer containing 0.5 mg/ml casein was added to block the glass surface for 5 min. Then 20 nM purified EGFP-tagged motors diluted in BRB80CA (BRB80, 0.2 mg/ml casein, 1 mM MgATP) were introduced into the chamber and allowed to adhere to the surface for 10 min. Finally, motility solution (BRB80, 10 µM taxol, 1 mM MgATP, 64 nM Alexa Fluor® 647-labeled microtubules and an oxygen scavenger system consisting of 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase and 0.5% β-ME) was flowed into the flow cell.
Microtubule gliding was monitored by epifluorescence microscopy on a Nikon TE2000 inverted microscope (60×, 1.45 N.A. objective). Fluorescence images were captured by a Photometrics® Cascade 512B CCD camera (Roper Scientific, Inc., Tucson, AZ), recorded onto computer and analyzed offline using the image acquisition and processing software MetaVue™ 6.1 (Molecular Devices Corporation, Downingtown, PA). The gliding velocities were measured by tracing the microtubule position using the tracking function in MetaVue™. The minimum detectable threshold was 0.2 µm.

### 3.2.4 Single molecule fluorescence assays

The motility of single, EGFP-tagged kinesin molecules was observed along AlexaFluor®647-labeled microtubule via the Nikon TIRF microscope (Nikon Instruments Inc., Melville, NY), an evanescent wave imaging system built on a Nikon TE2000 inverted microscope. The basic setup is shown in Figure 3.2. For illumination, an air-cooled 488 nm argon laser was used to excite EGFP-tagged motor molecules and a 633 nm He-Ne laser was used to excite AlexaFluor®647-labeled microtubules. The laser beam was introduced into a high numerical aperture objective (Plan Apo 60X/1.45 oil) via a TIRF attachment port in the side using optical fibers, ND filters, mirrors and focusing lenses. The incident angle of the laser beam projected via the objective into the cover glass can be adjusted by the laser position adjustment knobs. These knobs can move the laser beam focal point across the objective’s back focal plane. As the laser beam moves toward the edge of the plane, the beam is emitted at gradually sharper angles such that total internal reflection can take place at the glass-solution interface. Fluorescence signals were collected with the same objective as above and passed through
a filter cube consisting of an excitation filter, a dichroic mirror and an emission filter to isolate the fluorescence of interest (488 Line filter cube for EGFP fluorescence and 633 Line filter cube for Alexa Fluor® 647 fluorescence, Chroma Technology Corp, Rockingham, VT). After filtering, output signals were amplified and then captured by a Photometrics® Cascade 512B CCD camera (Roper Scientific, Inc.). The resulting images were recorded into computer and analyzed afterward using MetaVue™ 6.1 (Molecular Devices Corporation).

Samples for TIRF imaging were prepared according to the following procedures: i) flow cells were preloaded with BRB80 buffer containing 0.5 mg/ml casein to block the glass surface for 5 min, ii) 100 nM dead kinesin motors (from a bad motor prep) diluted in BRB80CA (BRB80, 0.2 mg/ml casein, 1 mM MgATP) were introduced into the chamber for 5 min, iii) 0.6 µM AlexFluor®647-labeled microtubules were flowed into the flow cells and immobilized onto glass surface via the above dead motors, iv) after 10 min, flow cells were washed twice by 50 µl of BRB12 buffer (12 mM Pipes, 1 mM EGTA, 2 mM MgCl₂, pH 6.8) plus 0.2 mg/ml casein and 10 µM taxol, v) EGFP-tagged motors were diluted to single molecule level (0.02 nM – 5 nM) in BRB12CT buffer (BRB12, 0.2 mg/ml casein and 10 µM taxol) supplemented with 2 mM MgATP, 20 mM D-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase and 0.5% β–ME. As an alternative immobilization method, microtubules were absorbed onto glass surfaces coated with 3-aminopropyltriethoxysilane (APTES) (Pierce Biotechnology, Inc., Rockford, IL).

Flow cells were placed on the oil-immersed objective with cover glass down, and 633 nm He-Ne laser was first turned on to illuminate AlexFluor®647-labeled
microtubules to find the TIR interface. Then the filter cube was switched and EGFP-tagged motors were imaged in the same field with 488 nm argon laser on. Images of microtubules under TIRF were captured before and after switching to EGFP illumination. Only motor runs on stationary microtubules were counted.

Figure 3.2 Schematic illustration of objective-based TIRF setup used in these experiments.

3.2.5 Analysis of processivity

To characterize interdomain coordination, the key parameter to measure is the run-length, defined as the distance a motor moves along the microtubule before detaching. Processivity can be presented as the mean run length. Motility was analyzed using MetaVue™ 6.1 (Molecular Devices Corporation). The run lengths, velocities and
association times were determined by marking the binding and dissociation events of a single kinesin-GFP molecule with the MetaVue tracking function. Only movements on long microtubules (10 – 20 µm) were counted to minimize the possibility that an EGFP-tagged motor molecule would dissociate from the microtubule simply because of running off the end.

The dissociation of a single kinesin molecule from the microtubule is a single exponential decay process (Block et al., 1990; Vale et al., 1996), \( y = y_0 \exp(-x/a) \). The cumulative probability can be described as Equation 3.2:

\[
P(x) = \frac{\int_{x_0}^{x} y_0 \exp(-x/a) \, dx}{\int_{x_0}^{\infty} y_0 \exp(-x/a) \, dx} = \frac{\exp(-x/a) - \exp(-x_0/a)}{-\exp(-x_0/a)} = 1 - \exp[(x_0 - x)/a], \quad \text{(Equation 3.2)}
\]

where \( x_0 \) is the lower limit of the run length. To determine the mean run length, we first calculated the cumulative probability distribution of the run lengths, which plots the fraction of run lengths shorter than a given value versus the run length. Then we performed a nonlinear least squares fitting of the cumulative probability distribution data to \( 1 - \exp[(x_0 - x)/a] \). The decay constant, \( a \), is the only fitted parameter, and gives the mean run length of the distribution. Compared to the fitting to a single exponential decay, this procedure is much more insensitive to data binning. Run lengths as short as 0.2 µm can be detected in our system and run lengths above 1 µm can be determined with the greatest degree of accuracy. For KHCΔtail-GFP, a considerable fraction of the measured run lengths greater than 1 µm, so we set \( x_0 \) to 1 µm to exclusively incorporate the most accurate data for the fit. For KIF3A/B-GFP, KIF3A/A-GFP and KIF3B/B-GFP, the run lengths were much shorter and \( x_0 \) was set to 0.2 µm, which represents the lower
detection limit of our system. All fitting was performed using SigmaPlot 7.0 (Systat Software, Inc., Point Richmond, CA).

The observed run lengths underestimate the true run length, because a moving fluorescence motor molecule disappears either when it dissociates from the microtubule or when it irreversibly photobleaches. Because both the dissociation and photobleaching are first-order processes following single exponential decays, the true dissociation rate constant can be corrected as 

\[
k_{\text{off}} = k_{\text{obs}} - k_{\text{bleach}}
\]

(Romberg et al., 1998; Thorn et al., 2000). 

\(k_{\text{obs}}\) is the observed dissociation rate constant and is the reciprocal of the association time of the motor on the microtubule, equal to observed run length divided by motor speed. 

\(k_{\text{bleach}}\) is the photobleaching rate, which can be determined by measuring the fluorescence intensity decay as a function of time of large assemblies of surface or microtubule-bound motors and fitting the data to single exponential decays. Hence the run length of the motor corrected by photobleaching can be calculated as Equation 3.3:

\[
RL = \frac{v}{RL_{\text{obs}}} - k_{\text{bleach}}
\]

(Equation 3.3)

where \(RL_{\text{obs}}\) is the observed mean run length and \(v\) is the average velocity.

Although this analysis has been used in the literature, it has one flaw: there are two EGFP in each molecule of these dimeric motors. Hence both EGFP fluorophores must photobleach before they are mistaken as a dissociation event. Hence, the actual run length will be smaller than that corrected by Equation 3.3.

3.3 RESULTS
3.3.1 Expression of recombinant KIF3A/B-GFP, KIF3A/A-GFP and KIF3B/B-GFP

It has been reported that kinesin’s free tail domain inhibits the motor activity (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000). To best characterize the motility of KIF3A/B in single molecule fluorescence assays, the tail domains after residue Tyr$_{602}$ in KIF3A and residue Ser$_{603}$ in KIF3B were deleted and replaced with EGFP fusion protein.

EGFP genes were inserted into wild-type and chimaeric KIF3A and KIF3B genes, and the KIF3A/B-GFP, KIF3A/A-GFP and KIF3B/B-GFP proteins were expressed and purified in the optimized and scaled up baculovirus expression system. Figure 3.3 is a fluorescence image of the $S_f9$ insect cells visualized by epifluorescence microscopy with FITC filter cube (Chroma Technology Corp) after a 60-hour co-infection with KIF3A-GFP virus and KIF3B-GFP virus. The green color of the cells indicates that most of the cells were infected and had EGFP proteins expressed, confirming our expression system is highly effective.

![Image of Sf9 insect cells after KIF3A/B-GFP expression](image)

**Figure 3.3** $S_f9$ insect cells after KIF3A/B-GFP expression. Cells were imaged under mercury lamp excitation with FITC filter cube.
After purification, KIF3A/B-GFP appeared as a single 107 kD band on SDS-PAGE with two subunits being the same size, consistent with sequence predictions (Figure 3.4). Chimaeric KIF3A/A-GFP and KIF3B/B-GFP homodimers have the same protein size and same expression level as wild-type KIF3A/B-GFP heterodimer (Figure 3.4).

![Figure 3.4 SDS-PAGE of purified KIF3-GFP motors. Lane 1, molecular weight markers; lane 2, eluate of wild-type KIF3A/B-GFP heterodimer; lane 3 eluate of chimaeric KIF3A/A-GFP homodimer; lane 4 eluate of chimaeric KIF3B/B-GFP homodimer.]

3.3.2 Gliding motility of KIF3-GFP motors

Motility of KIF3-GFP motors, including wild-type KIF3A/B-GFP and chimaeric KIF3A/A-GFP and KIF3B/B-GFP, were confirmed using a high-density microtubule gliding assay. Since the tail domains of these motors were deleted, anti-GFP antibodies were used to enhance the absorption of KIF3-GFP motors onto glass surfaces. Because the truncation is so distal to the motor domain, it was expected that the motility would be identical to full length KIF3 motors.

Initially, the quality of the microtubule gliding motility of wild-type KIF3A/B-GFP and chimaeric KIF3B/B-GFP was poor. There were numerous stalls during the microtubule movements, and there was a large variance in the velocity (Table 3.1).
Furthermore, the percentage of moving microtubules was very low. The average velocity of KIF3A/B-GFP was determined as 186 ± 86 nm/s, which agrees with the velocity of full length KIF3A/B (188 ± 38 nm/s). The velocity of KIF3B/B-GFP, 299 ± 101 nm/s, was smaller than that of full length KIF3B/B (409 ± 47 nm/s) but it was still within the range of the experiment error. For the KHCΔtail-GFP control, the gliding velocity was 665 ± 129 nm/s and the velocity of its full length construct was 815 ± 104 nm/s.

The bad motility of these KIF3-GFP motors motivated me to sequence all of the gene constructs for KIF3A and KIF3B. The sequencing results revealed that there were five nonsilent point mutations in KIF3A gene and two nonsilent point mutations in KIF3B. All of the nonsilent point mutations were fixed by site-directed mutagenesis and new KIF3-GFP motors were expressed and purified using the same baculovirus expression system (Figure 3.2). Surprisingly, after fixing the point mutations the new KIF3A/B-GFP, KIF3A/A-GFP and KIF3B/B-GFP motors could propel microtubules at roughly the same velocities, 571 ± 76 nm/s, 536 ± 87 nm/s and 558 ± 48 nm/s, respectively (Table 3.1). In contrast, for their full length constructs having the point mutations, KIF3B/B moved twice faster than KIF3A/B and ten times faster than KIF3A/A (as discussed in 2.3.3). It was established that replacing kinesin’s tail domains with EGFP fusion proteins did not affect motor motility. For example, the velocity of KIF3A/B-GFP (571 ± 76 nm/s) is consistent with that of full length KIF3A/B (574 ± 32 nm/s). This is also true for KIF3A/A-GFP and KIF3B/B-GFP (Table 3.1).
Table 3.1 Gliding velocities of KIF3-GFP motors

<table>
<thead>
<tr>
<th>Motor</th>
<th>Velocity (nm/s ± S.D.)</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF3A/B-GFP</td>
<td>571 ± 76</td>
<td>N = 45</td>
</tr>
<tr>
<td>KIF3A/A-GFP</td>
<td>536 ± 87</td>
<td>N = 98</td>
</tr>
<tr>
<td>KIF3B/B-GFP</td>
<td>558 ± 48</td>
<td>N = 42</td>
</tr>
<tr>
<td>KIF3A/B-GFP*</td>
<td>186 ± 86</td>
<td>N = 48</td>
</tr>
<tr>
<td>KIF3B/B –GFP*</td>
<td>299 ± 101</td>
<td>N = 36</td>
</tr>
<tr>
<td>KIF3A/B</td>
<td>574 ± 32</td>
<td>N = 97</td>
</tr>
<tr>
<td>KIF3A/A</td>
<td>566 ± 27</td>
<td>N = 108</td>
</tr>
<tr>
<td>KIF3B/B</td>
<td>549 ± 14</td>
<td>N = 114</td>
</tr>
<tr>
<td>KIF3A/B*</td>
<td>188 ± 38</td>
<td>N = 396</td>
</tr>
<tr>
<td>KIF3A/A*</td>
<td>42 ± 11</td>
<td>N = 22</td>
</tr>
<tr>
<td>KIF3B/B*</td>
<td>409 ± 47</td>
<td>N = 573</td>
</tr>
<tr>
<td>KHCΔtail-GFP</td>
<td>665 ± 129</td>
<td>N = 39</td>
</tr>
<tr>
<td>Hiskin</td>
<td>815 ± 104</td>
<td>N = 27</td>
</tr>
</tbody>
</table>

* Motors made before the point mutations were fixed

After mutations were fixed the motility increased 12.5-fold for KIF3A/A, 2.0-fold for KIF3A/B and 0.3-fold for KIF3B/B. The drastic increase in the speed of KIF3A/A suggests that the point mutations in the KIF3A gene were much more deleterious than those in the KIF3B gene. Among the five nonsilent point mutations in the KIF3A gene, three were in the head domain (Gly\textsubscript{121} to Glu\textsubscript{121}, Ala\textsubscript{210} to Val\textsubscript{210} and Pro\textsubscript{296} to Ser\textsubscript{296}), one in the coiled-coil (Lys\textsubscript{404} to Arg\textsubscript{404}) and one in the Myc tag. For the KIF3B gene, only one nonsilent mutation was in the head (Ala\textsubscript{34} to Val\textsubscript{34}) and the other one in the coiled-coil (Ala\textsubscript{465} to Val\textsubscript{465}). Hence, in the head domains that are known to be crucial for motility, there were six nonsilent mutations for chimaeric KIF3A/A motor, four for wild-type KIF3A/B motor and only two for chimaeric KIF3B/B.

3.3.3 Single molecule fluorescence measurements of KIF3-GFP motors

To circumvent potential problems involved in microtubule gliding assays such as absorption and aggregation, single molecule fluorescence assays were performed to
quantitatively assess the processivity of KIF3A/B by directly observing the movement of single EGFP labeled motor molecules along microtubules.

The photobleaching rate of KIF3-GFP motors was first quantified by measuring the fluorescence intensity decay as a function of time. Microtubules were attached to glass surface via dead kinesins (see 3.2.4) and a large number of KIF3-GFP motors were tightly immobilized onto the microtubules in the presence of AMP-PNP (non-hydrolyzable ATP analogue) and an oxygen scavenging system. The fluorescence data could be nicely fit to a single exponential decay function (Figure 3.5). The average photobleaching rate, determined as the average of the decay constants from the fit, was measured to be $0.039 \pm 0.006 \text{ s}^{-1}$.

![Figure 3.5](image.png)

**Figure 3.5** Photobleaching behavior of a population of KIF3A/B-GFP. Bleaching rate was determined by observing the fluorescence decay over time in a selected area where hundreds of KIF3A/B-GFP molecules were bound on a single microtubule. The fluorescence intensity was plotted as a function of time and the data were fit to a single exponential function, yielding the rate constant for bleaching.

To investigate the bleaching behavior of individual motors, KIF3-GFP motors were diluted to 0.5 nM and added into flow cells precoated with 0.5 mg/ml casein. The surface density of KIF3-GFP motors was less than 18 molecules/µm$^2$. The change of
intensity of individual fluorescent spots over time was monitored similarly as above. Most of the dots disappeared in a two-step bleaching fashion (Figure 3.6A) and less than 20% of the dots, which had much higher intensity, bleached gradually (Figure 3.6B). Since each dimeric motor molecule contains two EGFP, these results confirm that most of the KIF3-GFP motors exist as dimers and only a minor population of the motors aggregate. Based on the two-step bleaching curve, the intensity per single motor molecule was estimated to be 1200 – 1800 arbitrary fluorescence units. Any fluorescent dot with net intensity after background subtraction above 1800 was considered as aggregates and was excluded from the data analysis.

Figure 3.6 Photobleaching behavior of KIF3A/B-GFP at low surface density. The intensity of individual fluorescent spot (5 x 5-pixel area) was recorded and then plotted as a function of time. Fluorescent
molecules were excited with maximum laser power (1 mW). (A) Two-step bleaching at single molecule level. (B) Continuous bleaching for aggregates.

3.3.3.1 KIF3A/B-GFP is a processive motor, with a much shorter run length than KHCΔtail-GFP

When EGFP-tagged truncated conventional kinesin, KHCΔtail-GFP, was diluted to 0.02 – 0.5 nM and combined with immobilized AlexaFluor®647-labeled microtubules, the association and movement of numerous fluorescent KHCΔtail-GFP molecules could be clearly observed in the TIRF setup (Figure 3.7B). However when KIF3A/B-GFP was diluted to the same concentration and assayed under the same condition, only a few events of single motor molecules moving along microtubules were detected (Figure 3.7A), indicating the on-rate of KIF3A/B-GFP associating with microtubules was much slower than that of KHCΔtail-GFP. In order to obtain sufficient processive runs for quantitative data analysis, the concentration of KIF3A/B-GFP was increased to 5 nM. By doing this, the frequency of movements was greatly increased, but the background fluorescence was also elevated, making the process of data analysis more difficult than for KHCΔtail-GFP. For the old KIF3-GFP motor preps that contained the point mutations, very few moving events were observed at either low or high surface densities. All of the single molecule data presented here were from KIF3-GFP motors that had the point mutations fixed.

The average velocity of moving KIF3A/B-GFP spots was 200 ± 197 nm/s (N = 95), which is much slower than the velocity measured in microtubule gliding assay (571 ± 76 nm/s). However, the spots for KHCΔtail-GFP control moved at 732 ± 161 nm/s (N = 221), consistent with the microtubule gliding assay (665 ± 129 nm/s).
There are three systematic errors possibly contributing to the deviation of KIF3A/B-GFP’s velocity in single molecule fluorescence assay from that in gliding assay. Firstly, there is a 1 pixel uncertainty in defining the position of moving dots, which corresponds to 0.1 µm. Since the run length is 0.67 µm (Figure 3.9), the error is ~15%. Secondly, the two assays used different buffers, BRB12 for single molecule fluorescence assay and BRB80 for gliding assay. BRB12 has lower salt concentration than BRB80, which may cause slower stepping. Thirdly, in the single molecule fluorescence assay, motors may be partially unfolded and have lost some motility due to photodamage induced by laser illumination. In the gliding assay, even if some motors are denatured, the loss of motility of the weak motors can be overwhelmed by the strong ones because the motility is measured at multiple molecule level. There is another potential system error that can affect the run length but not the velocity. Motors tracking along the helically arranged microtubule protofilaments may have a chance to collide onto surface and detach. However, we do not believe this has a significant effect for our motors because the protofilament helical pitch (3 ~ 6 µm) is relative long compared to the average run length (0.67 µm) (Ray et al., 1993). Also in our single molecule fluorescence assay, the surface was coated with a layer of dead kinesin motors and then microtubules were attached via these dead motor linkers, so the observed motors may move on all sides of the microtubule rather than bumping into the surface.
Figure 3.7 Superimposed images of individual KIF3A/B-GFP (A) and KHCΔtail-GFP (B) (pseudo-colored green) moving along AlexaFluor®647-labeled microtubules (pseudo-colored red).

To determine the run length, the distance that fluorescent spots moved along the microtubule from the time that they bound to the time when they disappeared was measured. Initially, a histogram of the run length for KIF3A/B-GFP was plotted and fit by single exponential distributions. However, the processive runs were so short that the curve fitting showed an extensive dependence on data binning. For instance, for a bin width of 0.5 µm, a decay length of 0.26 µm was estimated from the fit (Figure 3.8A), but when the bin width was reduced to 0.25 µm, the estimated mean run length increased to 0.51 µm (Figure 3.8B).
Figure 3.8 Histograms of the run length of KIF3A/B-GFP and KHCΔtail-GFP. The data were fit by a single exponential function. The decay constant of the fit gives the mean run length of the motor molecule. (A) 0.5 µm bin width for KIF3A/B-GFP. (B) 0.25 µm bin width for KIF3A/B-GFP. (C) 1.0 µm bin width for KHCΔtail-GFP. (D) 0.5 µm bin width for KHCΔtail-GFP.

To avoid data binning, the fraction of motors moving with run lengths shorter than a certain value was calculated and plotted against the given run length, and then the data were fit to a cumulative probability distribution as described in 3.2.5. The mean run length for KIF3A/B-GFP was determined as the decay constant of the fit, which was 0.67 µm (Figure 3.9A). It was much shorter than the run length of KHCΔtail-GFP, 2.93 µm, determined by the same method (Figure 3.9B).
Figure 3.9 Cumulative probability distribution of the run length of KIF3A/B-GFP (A) and KHCΔtail-GFP (B). The data were fit to a cumulative probability function, $1 - \exp[(x_0-x)/a]$. $x_0$ is the lower limit for runs included in the analysis and is used to exclude shorter runs that are either undetectable ($x_0 = 0.2$ for KIF3A/B-GFP) or not measured with the greatest accuracy ($x_0 = 1$ for KHCΔtail-GFP). The decay constant of the fit, $a$, gives the mean run length of the motor molecule.

3.3.3.2 Homodimeric KIF3A/A-GFP is slightly more processive than homodimeric KIF3B/B-GFP

To investigate the role that each head plays during KIF3A/B’s processive runs, the processivity of two chimaeric homodimers, KIF3A/A-GFP and KIF3B/B-GFP, were characterized by TIRF microscopy using similar methods.

Like KIF3A/B-GFP, relatively high concentration of KIF3A/A-GFP and KIF3B/B-GFP (5 nM) were needed to enhance the possibility of motor-microtubule interaction for detecting sufficient processive runs (Figure 3.10).
Figure 3.10 Superimposed images of individual KIF3A/A-GFP (A) and KIF3B/B-GFP (B) (pseudo-colored green) moving along AlexaFluor®647-labeled microtubules (pseudo-colored red).

Fluorescent spots for KIF3A/A-GFP and KIF3B/B-GFP moved at approximately the same speed, 388 ± 205 (N = 83) nm/s and 358 ± 274 nm/s (N = 44), respectively (Table 3.2). Both were faster than KIF3A/B-GFP (200 ± 197 nm/s) measured under the same conditions. These speeds that we measured in single molecule fluorescence assays were slightly different from what we measured in microtubule gliding assays, but were within the range of variation typically observed for assays performed under different conditions.

Figure 3.11 shows the histograms of run length distribution for KIF3A/A-GFP and KIF3B/B-GFP. Due to the problem of data binning, the mean run lengths were instead determined by fitting to the cumulative probability distribution, yielding 0.77 µm for KIF3A/A-GFP and 0.58 µm for KIF3B/B-GFP (Figure 3.12). These results demonstrate that both chimaeric homodimers have similar run lengths as the wild-type heterodimer, suggesting for processivity two identical heads coordinate as well as two different heads.
Figure 3.11 Histograms of the run length of KIF3A/A-GFP (A) and KIF3B/B-GFP (B).

Figure 3.12 Cumulative probability distribution of the run length of KIF3A/A-GFP (A) and KIF3B/B-GFP (B). The data were fit to a cumulative probability function $1 - \exp[(x_0 - x)/a]$, with $x_0$ set to 0.2 µm. The decay constant of the fit, $a$, gives the mean run length of the motor molecule.

Based on the cumulative distribution function, the probability that a kinesin molecule will release from the microtubule rather than taking its next step can be estimated by the equation,

$$P_{off} / \text{step} = 1 - e^{(-d/a)}$$  \hspace{1cm} (Equation 3.4)
where $d$ is equal to 8 nm if we assume kinesin takes 8 nm steps, and $a$ is the mean run length. The probability of detachment per step was calculated to be 1.18% for wild-type KIF3A/B-GFP, 1.03% for chimaeric KIF3A/A-GFP and 1.37% for KIF3B/B-GFP (Table 3.2).

Since the two heads are identical in each of the chimaeric homodimers, we can infer that the A head have 1.03% possibility to detach per step and the B head have 1.37% possibility. If we assume that the performance of each head in the heterodimer is the same as in the context of homodimers, then the probability of detaching after each head in the heterodimer takes one step is estimated to be $1.03\% + 1.37\% = 2.40\%$, which is in good agreement with our finding of release probability for KIF3A/B-GFP taking two steps, $2 \times 1.18\% = 2.36\%$

**Table 3.2** Motility of EGFP-tagged kinesin in single molecule fluorescence assay

<table>
<thead>
<tr>
<th>Motor</th>
<th>Velocity (nm/s ± S.D.)</th>
<th>Sample Size</th>
<th>Runlength (µm)</th>
<th>Dissociation Rate (s⁻¹)</th>
<th>$P_{\text{off}/\text{step}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF3A/B-GFP</td>
<td>200 ± 197</td>
<td>$N = 95$</td>
<td>0.67</td>
<td>0.299</td>
<td>1.18%</td>
</tr>
<tr>
<td>KIF3A/A-GFP</td>
<td>388 ± 205</td>
<td>$N = 83$</td>
<td>0.77</td>
<td>0.504</td>
<td>1.03%</td>
</tr>
<tr>
<td>KIF3B/B-GFP</td>
<td>358 ± 274</td>
<td>$N = 44$</td>
<td>0.58</td>
<td>0.617</td>
<td>1.37%</td>
</tr>
<tr>
<td>KHCΔtail-GFP</td>
<td>732 ± 161</td>
<td>$N = 187$</td>
<td>2.93</td>
<td>0.250</td>
<td>0.27%</td>
</tr>
</tbody>
</table>

### 3.4 DISCUSSION

In this chapter we quantitatively assessed the processive behavior of KIF3A/B, the mouse ortholog of Kinesin-2, by engineering EGFP fusion proteins of KIF3A/B and then observing the movement of fluorescent KIF3A/B-GFP molecules along microtubules. It was demonstrated that KIF3A/B is processive but is only one-fourth as processive as conventional kinesin. Two chimaeric homodimers, KIF3A/A-GFP and KIF3B/B-GFP were investigated in the same single molecule fluorescence assay and the
results showed that homodimeric KIF3A/A is slightly more processive than homodimeric KIF3B/B.

We found that there were three nonsilent point mutations in the KIF3A motor domain and one in the KIF3B motor domain, randomly picked up during DNA construction. And these mutations were confirmed to exist in the constructs that we generated in chapter 2 as well. After all the point mutations were fixed, the ten-fold velocity difference between the two heads as we observed in chapter 2 vanished and all the three constructs KIF3A/B, KIF3A/A and KIF3B/B had approximately the same microtubule gliding velocity, 574 ± 32 nm/s, 566 ± 27 nm/s, and 549 ± 14 nm/s, respectively. The speeds of KIF3-GFP that we measured in single molecule fluorescence assays were slightly different from what we measured in microtubule gliding assays, but within the range of variation typically observed for assays performed under different conditions.

An early study on KIF3A done by Kondo et al showed that both recombinant KIF3A homodimer and native KIF3A protein, actually native KIF3A/B heterodimer as determined later, could move microtubules at the same velocity of 0.6 µm/s (Kondo et al., 1994). Since their recombinant motor proteins were purified simply by microtubule pelleting, the observed motility were probably due to native kinesin contaminants from the Sf9 host cells. Later on, the same group reported that recombinant KIF3A/B had the same microtubule gliding velocity as a KIF3B/B chimaera, which is 0.3 µm/s (Yamazaki et al., 1995). Though the above velocity data were not consistent, their work at least showed that both heads could move at similar speeds to wild-type KIF3A/B no matter what speed it is. Pierce et al reported that KRP85/95-GFP, the sea urchin ortholog of
Kinesin-2 had gliding velocity of 202 nm/s (Pierce et al., 1999), slower than the velocity of 410 nm/s observed for the native Kinesin-2 heterodimer purified from sea urchin eggs (Cole et al., 1993). The velocity difference between KRP85/95-GFP and our KIF3A/B-GFP was likely due to the species difference. Another plausible explanation is that the motility of KRP85/95-GFP was measured on axoneme, a 9+2 microtubule bundle formed by microtubules and associated proteins, and the motor performance on axonemes may be different from that on cytoplasmic microtubules.

After the point mutations were fixed, there was no significant change in the speed of KIF3B/B but there was a dramatic increase in KIF3A/A (~ 12 fold). To find out how the mutation of three amino acids in each head could drastically reduce the motility of KIF3A/A, we evaluated the potential influence of each mutation by mapping the mutation site onto 3D structure of the protein. Because the crystal structure of KIF3A/B has not been resolved, the sequences of KIF3A and KIF3B were aligned and mapped to the crystal structure of conventional kinesin (Sack et al., 1999; Sack et al., 1997). We found that in the KIF3A head, the sites where the three nonsilent point mutations occurred are within the regions that are highly conserved among KIF3A, KIF3B and conventional kinesin from human and rat. After sequence alignment and structure mapping, the three point mutations in KIF3A head, Gly121 to Glu121, Ala210 to Val210 and Pro296 to Ser296, were respectively matched to Gly106, Val194 and Pro276 in human kinesin and Gly107, Val195 and Pro278 in rat kinesin (Figure 3.13). Gly107 is located in loop 5 of rat kinesin’s 3D structure, a region surrounding the nucleotide binding pocket. Val195 is positioned in loop 9 and this loop is immediately upstream of the switch I helix (α3a helix in Figure 3.11) which directly interacts with the ATP molecule inside the nucleotide
binding core. The residue Pro$_{278}$ is situated in loop 12, which is the primary microtubule-binding element.

![Figure 3.13](image)

**Figure 3.13** Structure of conventional kinesin from *rattus norvegicus (PDB # 2KIN)*. Structures interacting with microtubules are highlighted as green ($\alpha4$ - L12 - $\alpha5$ and $\beta5a$ - L8 - $\beta5b$) and the nucleotide binding cores are highlighted in dark blue (N1-4). The arrows are pointed to the three amino acids that are corresponded to the three nonsilent point mutations in the KIF3A head.

It was reported that mutations either in the microtubule-binding core or in the nucleotide binding pocket could dramatically reduce kinesin speeds (Kaseda et al., 2002; Kaseda et al., 2003; Woehlke et al., 1997). Kaseda et al mutated three residues (Tyr$_{274}$Ala_Arg$_{278}$Ala_Lys$_{281}$Ala) in loop 12 of human conventional kinesin, disrupting the microtubule-binding site. They reported that the mutant homodimer L12/L12 had neither measurable microtubule-activated ATPase nor microtubule gliding activity, and the mutant heterodimer WT/L12 (101 nm/s) was substantially slower than
that of the wild-type (679 nm/s) (Kaseda et al., 2002). In another paper, Arg{sub}14 of human conventional kinesin was mutated to alanine, which altered nucleotide-binding motif N-4. The ATPase rate and the microtubule gliding speed (36 nm/s) of mutant homodimer were considerably slower than the wild-type (636 nm/s) (Kaseda et al., 2003). The velocity of mutant heterodimer WT/Arg{sub}14Ala was 86 nm/s, only 13.5% that of the wild-type (Kaseda et al., 2003).

For our mutations, Gly{sub}121Glu and Ala{sub}210Val reside near the nucleotide binding pocket, but do not directly interact with ATP or ADP molecule. Hence, they are not as injurious as Arg{sub}14Ala generated by Kaseda et al in terms of altering the kinetics of nucleotide binding and release. I believe that the mutation of Gly{sub}121Glu is more critical than Ala{sub}210Val simply because in Gly{sub}121Glu neutral glycine and negatively charged glutamic acid are less related, compared to alanine and valine as a pair in Ala{sub}210Val. The mutation of Pro{sub}296Ser is located exactly in the core of microtubule-binding interface. Therefore, this mutation is more critical than the other two (Gly{sub}121Glu and Ala{sub}210Val). For mutation Pro{sub}296Ser, a hydrophobic proline is mutated to a hydrophilic serine. It’s unknown how this type of mutation can alter the microtubule binding though we know that Kaseda et al’s mutation of primarily positively charged residues to neutral (Tyr{sub}274Ala_Arg{sub}278Ala_Lys{sub}281Ala) can disrupt the electrostatic interaction with the negatively charged tubulin molecule.

Though the three point mutations in KIF3A head occurred in the regions that are associated with nucleotide and microtubule binding, it is still mysterious how these mutations alter the kinetics of nucleotide and microtubule binding.
Similarly, we mapped the sequence of the KIF3B head to the crystal structure of conventional kinesin. The residue where the only mutation occurred (Ala$_{34}$ to Val$_{34}$) was matched to Lys$_{32}$ in both human and rat kinesin. The regions near this residue are not conserved among KIF3A, KIF3B and conventional kinesin, and they are far away from important sites such as nucleotide binding pocket, microtubule binding core and neck-linker. In addition, changing alanine to valine only involves adding two methyl groups, which is a subtle difference. Therefore, the mutation of Ala$_{34}$Vla in KIF3B head is not expected to have a significant effect on motility, consistent with our experimental results.

By using the TIRF microscopy, we obtained the first quantitative processive run length measurements for Kinesin-2 heterodimers (0.67 µm). This finding of processivity contrasts with work from Pierce et al who failed to measure processive runs of KRP85/95 in single molecule fluorescence assays (Pierce et al., 1999). A reasonable explanation for the lack of processivity is that the tail domains of their KRP85/95 were not deleted so that the motility of full-length KRP85/95 in solution was inhibited by its tail domains in the absence of cargo binding, similar to conventional kinesin (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000). We found that the run length of KIF3A/B was much shorter than that of truncated conventional kinesin KHC$\Delta$tail. The run length we obtained for Drosophila conventional kinesin (2.93 µm) is longer than what other people reported for human conventional kinesin. Three papers from the Vale lab reported that human K560-GFP, a construct similar to our Drosophila KHC$\Delta$tail-GFP but including one more amino acid, had a processive run length of 1.33 µm, 1.5 µm, and 1.29 µm (Pierce et al., 1999; Romberg et al., 1998; Thorn et al., 2000). However their processive runs were observed along axonemes instead of microtubules, which may make the
difference. The average velocity of their fluorescent motors along axonemes was around 0.35 µm/s, only one half of what we measured along microtubules. In another paper, Lakamper et al reported a run length of 0.83 µm for K560-Cy3, a truncated human conventional kinesin chemically labeled by organic dye Cy3 (Lakamper et al., 2003).

Is it possible that the observed long run length for our conventional kinesin is simply due to motor aggregates? The answer is no. The fact that most fluorescent dots on the surface showed characteristic two-step bleaching behavior demonstrated majority of our motors were in form of single molecules. Any moving dot with higher fluorescence intensity than those bleached in two-step fashion was not incorporated into the data analysis, excluding the possibility that the single spot motility observed for KHCΔtail-GFP and KIF3-GFP motors was produced by motor aggregates instead of single motor molecules.

As discussed in 3.2.5, the observed disappearance of EGFP-tagged kinesin molecules off a microtubule is the sum of two independent exponential processes, dissociation of motors from the microtubule and irreversible photobleaching of EGFP molecules. The run length can be corrected by the equation \( RL = \frac{v}{\frac{v}{RL_{obs}} - k_{\text{bleach}}} \), where velocity \( (v) \), observed run length \( (RL_{obs}) \) and photobleaching rate \( (k_{\text{bleach}}) \) have already been determined. After correction, the run length was calculated to be 0.77 µm for KIF3A/B-GFP, 0.86 µm for KIF3A/A-GFP, 0.62 µm for KIF3B/B-GFP and 3.47 µm for KHCΔtail-GFP. But since there were two EGFP fluorophores in a single kinesin molecule, the above correction overestimated the true run length. Therefore, the true run length should be in the middle of the observed value and the corrected value, and the effect of photobleaching on our EGFP-tagged KIF3 motors was negligible.
Sequence alignment and structure mapping suggests that the two head domains of KIF3A/B only share 69% sequence identity, and there are divergences surrounding the neck linker interaction region, nucleotide binding pocket, and microtubule binding region. Therefore, although the two heads had similar moving speeds after fixing the mutations, we still believe that there are other functional differences between the two heads. If the two heads of KIF3A/B are biochemically tuned to complement one another, then the heads are expected to have different degrees of processivity and microtubule affinity. We have measured that the processive runs of the KIF3A/A homodimer (0.77 µm) are slightly longer than the KIF3B/B homodimer (0.58 µm), but the difference is not significant. In the single molecule fluorescence assays, we found that the number of KIF3A/A-GFP molecules binding to and then moving along a microtubule in a given time was much larger than those of KIF3B/B-GFP and KIF3A/B-GFP, though all of them were smaller than that of conventional kinesin. Thus, we interpret this to mean the A head has a much higher on-rate than the B head. In addition, we have determined the off-rate ($k_{off}$) of the KIF3A/A homodimer (0.504 s$^{-1}$), slightly smaller than that of the KIF3B/B homodimer (0.617 s$^{-1}$). Combining these two rates and using the relationship of $K_D = k_{on} / k_{off}$, we can conclude that the A head of KIF3A/B has a much higher microtubule-binding affinity than the B head. This affinity is quantified by a method described in chapter 4, called fluorescence correlation spectroscopy (FCS).
4.1 INTRODUCTION

Our previous data indicated that the A head had higher microtubule affinity than the B head because for the KIF3A/A homodimers we observed a higher on-rate and a lower off-rate. In this chapter, we explore novel fluorescence techniques to quantitatively measure the microtubule affinity of Kinesin-2 motors. The goal is both to better characterize the fluorescently labeled KIF3 motors to understand their function, and to develop a novel high resolution method for analyzing motor-microtubule interactions free in solution.

Historically, the equilibrium dissociation constant $K_D$ (the inverse of affinity) for motor-microtubule binding are measured in the microtubule pelleting assay, in which the fraction of motors that co-sediment with microtubules is determined at a range of microtubule concentrations. However, the technique is complicated by inactive motors and errors in quantifying protein levels from gels. Also it is limited by its sensitivity because motor proteins at low concentrations cannot be detected on gels and nonprocessive motors cannot bind sufficiently for gel detection. Another method for determining $K_D$ is to measure ATPase kinetics and the microtubule concentration at half maximal rate represents $K_D$. However, this is an indirect method.

We approached this measurement in two novel ways, by quantitatively analyzing the rotational diffusion using time-resolved fluorescence anisotropy and the translational diffusion using fluorescence correlation spectroscopy (FCS) for EGFP-tagged motors in the presence of varying microtubule concentrations. This work was a collaboration with
Dr. Ahmed A. Heikal and his student Deepa J. Muldaliar (The Pennsylvania State University).

4.1.1 Time-resolved fluorescence anisotropy

Thanks to the recent availability of ultra-fast laser sources and detectors, the technique of time-resolved fluorescence anisotropy has become a powerful tool for detecting rapid rotational motion of molecules on nanosecond time scales with high sensitivity. This technique provides structural information of individual molecules and molecule-molecule interactions beyond the capability of steady-state fluorescence measurements.

The basic principle behind the time-resolved fluorescence anisotropy can be described as follows. When a pulse of a linearly polarized light of a certain wavelength passes through a sample, only fluorescent molecules with transition dipole moment oriented parallel to the polarization plane of the excitation light are preferentially excited, while those aligned perpendicularly are not excited at all. Immediately after excitation, the excited molecules rotate randomly due to Brownian rotational diffusion and then decay to the ground state by fluorescence. As a result, the fluorescence signal is depolarized. The randomized orientation of a fluorophore at the moment of fluorescence emission determines the polarization plane of the emitted photon. By placing polarizers in front of the detector, we can resolve parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) polarizations with respect to the polarization plane of the excitation light. The degree of polarization of the emitted fluorescence can be described by a physical quantity called, anisotropy $r$, where 

\[ r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \] (Lakowicz, 1999).
In the simplest case, the anisotropy can be fit to a single exponential decay. The rotational time, defined as the decay time constant, is dependent on the hydrodynamic volume of the fluorophore and the viscosity of the surrounding environment. However, if each fluorophore exists in a number of different states (free, bound or other), the anisotropy may have a multi-exponential decay, and the fractional contribution of each component to the total emission can be obtained as one of the fit parameters of the decay curve. Hence, in addition to molecular orientation, rotational mobility and molecule size, this technique can be used to investigate the interaction between molecules such as ligand-receptor binding and protein-protein binding. For examples, Smith et al applied time-resolved fluorescence anisotropy to the investigation of the adsorption of the dye Rhodamine B and a Rhodamine B-labeled cationic polyelectrolyte onto colloidal silica (Smith et al., 1998). Bailey and coworkers used this technique to study the interaction of dansyl-labeled DNA primer with the Klenow fragment of \textit{E. coli} DNA polymerase I (Bailey et al., 2001). To date, this technique has not been applied to studying motor proteins. In this chapter, we investigate the structural flexibility of EGFP-Tagged KIF3A/B motor using time-resolved fluorescence anisotropy and evaluate the feasibility of this technique in determining the binding affinity of motor-microtubule interaction.

4.1.2 Fluorescence correlation spectroscopy (FCS)

The concept of fluorescence correlation spectroscopy (FCS) was initially introduced in the early 1970s by Webb and coworkers (Magde et al., 1972). Following the development of the electronics, computer, optics and lasers, FCS has been widely spread into the study of biological sciences in the last decade. It can measure the
physical and chemical properties of dilute (~1 nM) fluorescent species in solution with single molecule sensitivity (Hess et al., 2002).

FCS is based on the measurement of fluctuations of the fluorescence due to the diffusion of the fluorophore in and out of the excitation volume or to a change of fluorescence quantum yield caused by chemical reactions. The excitation volume is confined to the order of 1 femtoliter.

The fluorescence fluctuations provide information on the diffusion rate of a fluorescent molecule, which in turn is directly dependent on the molecule’s mass. Consequently, any increase in the mass of a biomolecule, for example as a result of interaction with a second molecule, can be detected as an increase in the molecule's diffusion time. Hence, in addition to the applications of determining the translational diffusion coefficient, the size and the absolute concentration of a molecule (Elson and Webb, 1975; Magde et al., 1972; Weissman et al., 1976), FCS is an ideal approach for the study of kinetic features of molecular interactions in solution (Bonnet et al., 1998; Rauera et al., 1996; Schwille et al., 1997). In this chapter, we used FCS to determine the binding affinity of KIF3A/B motors to microtubules.

4.2 MATERIALS AND METHODS

4.2.1 Time-resolved fluorescence anisotropy

4.2.1.1 Sample preparation

Since this experiment and the next FCS experiment were done before the point mutations in KIF3 genes were discovered, the KIF3A/B-GFP motors used in this chapter were the ones that contained those point mutations.
Unlabeled tubulins were polymerized into microtubules with very high concentration (~115 µM). KIF3A/B-GFP motors (1 µM) were diluted into BRB80 buffer and incubated at room temperature for at least 30 min. To remove aggregated motors, the sample was centrifuged at 30 psi for 10 min in a Beckman air-driven ultracentrifuge and the supernatant was collected. The resultant KIF3A/B-GFP motors were then combined with microtubules in the presence of 2 mM ATP and 20 µM taxol in six different tubes, where the six samples had a sample volume of 100 µl, a constant motor concentration of 0.25 µM and varying microtubule concentration of 0 µM, 0.1 µM, 0.3 µM, 1 µM, 3 µM and 10 µM, respectively. To make a sample for assaying motors in the bound state (motor-microtubule complexes), 0.25 µM of KIF3A/B-GFP were first mixed with 0.25 µM of microtubules in the presence of 1 mM AMP-PNP and 20 µM taxol. Then after 10 min incubation at room temperature, the sample was airfuged for 10min, the pellet was washed with 100 µl of BRB80 supplemented with 20 µM taxol and resuspended in BRB80 containing 1 mM AMP-PNP and 20 µM taxol. All the samples were incubated for 30 min at room temperature before being added into mini petri dishes and observed under the water-immersed objective of the microscope.

4.2.1.2 Experimental setup

A mode-locked Ti:Sapphire laser (Coherent MIRA 900-F) was operated to produce femtosecond short pulses with a repetition rate of 76 kHz (Figure 4.1). The wavelength was tuned to 960 nm. A second harmonic generator (SHG) was used to reduce the wavelength to 480 nm for EGFP excitation and confine the excitation volume. A pulse picker, electro-optic modulator (EOM), was added for picking the highest intensity pulse out of the pulse train. After that, the pulsed laser was split into two
beams. One laser beam was used to trigger a photodiode and the timing signal was sent into a single photon counting device, and the other laser beam was directed into the sample on the IX81 inverted microscope from Olympus. The emission fluorescence was collected by the objective and then filtered by a FITC filter cube. Afterwards, the emission fluorescence was simultaneously passed through two polarizers that were oriented as a T-format, and then respectively detected by two multichannel plates (MCP). The output of the MCP was detected by a single photon counting device where the time-resolved fluorescence decay was acquired by time-correlated single photon counting (TCSPC).

Due to the low level and highly repetitive signals, the fluorescence intensity was usually so low that in each single laser pulse, no more than one single photon is emitted and detected. Inside the TCSPC device, the arrival time of a photon detected by the MCP relative to the trigger pulse generated by repetitive excitation pulses was recorded by a time-to-amplitude (TAC) converter, in which the output of voltage was proportional to the photon arrival time. This voltage was then sent to a multichannel analyzer (MCA), which sorted the TAC signal by voltage with the MCA channel number proportional to photon arrival time. The output of the MCA was recorded into a computer via a PC card. Therefore, after many photons emitted by repetitive laser excitation, a histogram of photon detection time built up, representing the fluorescence decay.
4.2.1.3 Data analysis

Two types of fluorescence decay were measured. First, fluorescence lifetime decay was recorded with the emission polarizer oriented at a 54.7º angle (magic angle) with respect to the excitation polarization plane. At this magic angle, complete loss of anisotropy occurred and the total intensity was measured regardless of the polarization of the sample (Equation 4.1).

\[
I_{\text{total}} = I_{54.7º} = I_{\parallel}(t) + 2I_{\perp}(t)
\]  

(Equation 4.1)

For a heterogenous population of fluorophores, the fluorescence lifetime decay can be represented as a multi-exponential decay (Equation 4.2):

\[
I(t) = I_0 \sum_{i=1}^{M} \alpha_i \exp(-t/\tau_i)
\]

(Equation 4.2)

where \(I_0\) is the initial fluorescence intensity, \(M\) is the number of decay components and \(\alpha_i\) and \(\tau_i\) are the fractional amplitude and fluorescence lifetime for the \(i\)th decay component,
and $\sum_{i=1}^{M} \alpha_i = 1$. For simple cases, the fluorescence intensity decay can be expressed as a single exponential decay (Equation 4.3):

$$I(t) = I_0 \exp(-t / \tau_f)$$  \hspace{1cm} (Equation 4.3)

Second, though the fluorescence anisotropy decay can’t be measured directly, it can be calculated from the emitted fluorescence intensity recorded through the T-format polarizers parallel and perpendicular to the incident light using Equation 4.4.

$$r(t) = \frac{I_{||}(t) - GI_{\perp}(t)}{I_{||}(t) + 2GI_{\perp}(t)}$$  \hspace{1cm} (Equation 4.4)

where G is the G-factor defined as the sensitivity ratio of the detection system to parallel and perpendicular polarizations.

For a homogeneous population of fluorophores, the time-resolved fluorescence anisotropy can be described as a sum of exponential decays (Equation 4.5).

$$r(t) = \sum_{j=1}^{N} r_j \exp(-t / \phi_j)$$  \hspace{1cm} (Equation 4.5)

where $N$ is the number of decay components, $r_j$ and $\phi_j$ are the anisotropy amplitude and rotational time for the $j$th decay component, and $\sum_{j=1}^{N} r_j = r_0$. For a sample of KIF3A/B-GFP motors existing in two states, free in solution and bound to microtubules, the anisotropy decay can be represented as a sum of decays contributed by the two populations using the law for the additivity of anisotropies if we assume the fluorescence lifetime of GFP remains the same in these two states (Equation 4.6).

$$r(t) = y \sum_{i=1}^{M} \beta_i \exp(-t / \phi_i) + (1 - y) \sum_{j=1}^{N} r_j \exp(-t / \phi_j)$$  \hspace{1cm} (Equation 4.6)
where $y$ is the molar fraction of free motors in the sample, $M$ is the number of decay components contributed by free motors and $\beta_i$ and $\phi_i$ are the anisotropy amplitude and rotational time for the $i$th decay component contributed by free motors; $1 - y$ is the molar fraction of bound motors in the sample, $N$ is the number of decay components contributed by bound motors, $r_j$ and $\phi_j$ are the anisotropy amplitude and rotational time for the $j$th decay component contributed by bound motors.

4.2.2 Fluorescence correlation spectroscopy

4.2.2.1 Sample preparation

Samples were prepared following the same protocol as used in the experiment of time-resolved fluorescence anisotropy, except that the final concentration of KIF3A/B-GFP motors in each sample was 20 nM instead of 0.25 µM.

4.2.2.2 Experimental setup

The FCS setup (from Dr. Erin Sheets’s lab in the Pennsylvania State University) was built on an inverted Nikon TE2000 microscope. A stable 488 nm argon laser was used to excite fluorophores inside the observation volume. Depending on the beam quality and diameter of the laser, a beam expander might be inserted to achieve a small sample volume before the laser beam was coupled into the microscope (Figure 4.2). The laser beam was reflected into an objective lens via a diachronic mirror and focused on the fluorescent sample, defining an excitation volume on the femtoliter scale. The fluorescence light from the sample was collected by the same objective and passed through the dichroic mirror and the emission filter. A pinhole in the image plane blocked any fluorescence light not originating from the focal region, thus providing axial
resolution. After passing through the pinhole, the emission light was focused onto the detector. Its output was connected to an autocorrelation card and the data were stored in a computer for later analysis.

![Experimental setup for FCS.](Image)

**Figure 4.2** Experimental setup for FCS.

### 4.2.2.3 Autocorrelation analysis

The fluorescence intensity $F(t)$, which varies over time due to fluctuations in the number of fluorescent molecules resident in the observation volume, was automatically correlated via an autocorrelation card. The normalized autocorrelation function can be described as (Maiti et al., 1997; Webb, 2001):

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}$$  \hspace{1cm} (Equation 4.7)

where $\langle F(t) \rangle$ is the average fluorescence, $\delta F$ is the deviation from the average fluorescence at time $t$ or a time interval $\tau$ later, the fluorescence deviation at time $t$, $\delta F(t)$, is compared to (multiplied with) the fluorescence deviation at time $t + \tau$ and this product
is averaged over all possible pairs of intensities that can be found in a long measurement session.

The autocorrelation function can be related to the diffusion time $\tau_D$, concentration ($N$ / observation volume), and the diffusion coefficient $D$ of the molecules of interest, by Equation 4.8 (Aragon and Pecora, 1976; Eigen and Rigler, 1994).

$$G(\tau) = \frac{1}{N} f(\tau / \tau_D)$$  \hspace{1cm} (Equation 4.8)

with $$f(\tau / \tau_D) = \left( \frac{1}{1 + \tau / \tau_D} \right)^{\frac{1}{2}} \left[ \frac{1}{\left(1 + \left(\frac{\omega_0}{z_0}\right)^2 \frac{\tau}{\tau_D}\right)} \right]$$

where $N$ is the average number of fluorescent molecules in the observation volume, and $\omega_0$ and $z_0$ are the lateral and axial beam waists. The relation between $\tau_D$ and $D$ is given by $\tau_D = \omega_0^2 / 4D$.

When the fluorescent molecule also binds to a target with sufficiently different diffusion coefficient (a factor of >2), the correlation function can be described as a sum of two fractions that diffuse independently (Equation 4.9) (Meseth et al., 1999; Van Craenenbroeck and Engelborghs, 1999):

$$G(\tau) = \frac{1}{N} \left[ (1 - y) \times f(\tau / \tau_{\text{free}}) + y \times f(\tau / \tau_{\text{bound}}) \right]$$  \hspace{1cm} (Equation 4.9)

where $1 - y$ and $y$ are the fractions of the free and bound states of the fluorescence molecules, respectively and $\tau_{\text{free}}$ and $\tau_{\text{bound}}$ are the diffusion times of free and bound fluorescence molecules.

In our FCS application, the main observable is changes in the motor diffusion time due to microtubule binding. To a first approximation, the diffusion constant is proportional to the cube root of mass, meaning this measurement is not strongly sensitive
to small changes in mass, but dimeric KIF3A/B-GFP fusion constructs have a molecular weight of roughly 220 kD, while a 2 µm microtubule has a molecular weight of roughly 350,000 kD. Hence, binding a microtubule should easily provide a >10-fold change in $D$.

It has been established that two molecular systems can be distinguished if their diffusion time differ by ~1.6 (Meseth et al., 1999).

To determine the equilibrium dissociation constant, $K_D$, we mixed 20 nM KIF3A/B-GFP with varying concentrations of microtubules from 0.1 µM to 10 µM tubulin. The autocorrelation function of these mixtures was analyzed in terms of the presence of two diffusing species (Equation 4.9), and the fractions of free KIF3A/B-GFP motors and microtubule-bound KIF3A/B-GFP motors at each microtubule concentration can be obtained by a two-component fit of this function. The $K_D$ values (expected to be in the 100 nM to 1 µM range) can be determined as the microtubule concentration at which half of the motors are free and half bound to the microtubule.

4.3 RESULTS AND DISCUSSION

4.3.1 Time-resolved fluorescence anisotropy

4.3.1.1 Fluorescence lifetime decay

The fluorescence decay of a homogenous population of fluorophores is usually a single exponential function. But for a more complex system containing a mix of different local environments surrounding the fluorophores, multi-exponential decays are often yielded. In addition, some non-radiative processes such as internal conversion and phosphorescence can compete with fluorescence emission for relaxation of excited state electrons to the ground state.
The fluorescence lifetime is the characteristic time that a fluorescent molecule remains in an excited state before returning to the ground state. The fluorescence lifetime measurements were conducted by measuring fluorescence decay immediately after a brief pulse of excitation. The fluorescence decays of EGFP tagged KIF3A/B motors (KIF3A/B-GFP) either free in solution or bound to microtubules were collected by setting the polarizers in front of the MCP detectors at magic angle. The rate of photon flux into the constant fraction discriminator (CFD) of the TCSPC device was adjusted to around 10000 photons/s and the data acquisition was stopped when the peak count in one of the MCA channels reached 8000 photons. The fluorescence lifetime was obtained for each sample by fitting the fluorescence intensity data to either a single exponential decay or a bi-exponential decay.

Our data showed that the fluorescence lifetime of EGFP molecules (2.66 ns) didn’t change when they were fused to KIF3A/B motors (2.64 ns) (Figure 4.3). When KIF3A/B-GFP motors were bound to different concentrations of microtubules in the presence of 2 mM ATP, the fraction of free motors in solution decreased as microtubule concentration increased, but the fluorescence lifetime maintained a similar value regardless of the microtubule concentration (Figure 4.3). Usually, when the molecular structure and local environment surrounding the fluorophore such as pH and concentration of ions are altered, a different fluorescence lifetime will be expected. The fact that there were no changes in the fluorescence lifetime of EGFP when it was fused to motors or when these motors were bound to microtubules in the presence of ATP demonstrates that the β-barrel protection of the embodied chromophore of EGFP is preserved during EGFP-motor fusion and motor-microtubule binding.
Figure 4.3 Normalized Fluorescence lifetime decays of KIF3A/B-GFP at varying microtubule concentrations. The data were fit to a single exponential decay or a bi-exponential decay. The fluorescence lifetime was then determined as the decay time constant of the fit.

An exceptional case was when KIF3A/B-GFP motors were completely bound to microtubules in the presence of AMP-PNP. In this case, the fluorescence decay followed a multi-exponential decay having a very fast decay component (tens of ps) with a significant relative amplitude in addition to the slow components (ns) (pointed by the arrow in Figure 4.3). This initial sharp decay was not due to detection of scattered excitation light because the position of this fast decay component was after the time zero that matched the peak of the system response function. This conclusion was also confirmed by repeating the experiment at different excitation intensities. The relative ratio of the amplitudes of the fast and slow decay components was independent of the excitation intensity. Therefore, the fast decay component in KIF3A/B-GFP-microtubule complex was real. In the presence of AMP-PNP, a nonhydrolyzable ATP analog, motors are tightly immobilized onto microtubules most likely with the two head domains
spanning 8 nm on two adjacent tubulin dimers (Hirose et al., 1997; Uemura et al., 2002). In contrast, in the presence of ATP, motors walk along microtubules with the two heads alternately stepping 16 nm past each other, which might result in a more flexible conformation in the tail domains where the EGFP fluorophores exist. A possible explanation for the fast component is that the tight binding of KIF3A/B-GFP onto microtubules in the presence of AMP-PNP somewhat denatured the EGFP, reducing the constraint of the β-barrel to the enclosed chromophore. Less constraint on the chromophore may result from activated isomerization reaction of the chromophore, a non-radiative process that competes with fluorescence to reduce the real fluorescence lifetime, generating a fast component in the fluorescence decay. Another plausible explanation is that in the sample with AMP-PNP, the molar ratio of motor to tubulin was set to one so that all motor binding sites (one for each tubulin dimer) on the microtubule were occupied by KIF3A/B-GFP. This crowding effect may cause homoFRET quenching between adjacent EGFP dimers, another non-radiative process that competes with fluorescence to reduce fluorescence lifetime.

4.3.1.2 Fluorescence anisotropy decay

To obtain time-resolved anisotropy decays for free and microtubule-bound KIF3A/B-GFP motors, fluorescence decays were collected simultaneously by two polarization channels: one parallel to the polarization plane of the excitation beam and the other perpendicular. The photon flux rate in the CFD from the above two channels was tuned to be around 20000 photons/s. The data acquisition was stopped when the peak photon counts for the parallel channel and perpendicular channel were around 50000 and 10000, respectively. The anisotropy decays can be calculated from Equation
4.4. The G-factor of our experimental setup was determined by recording the fluorescence decays of fluorescein simultaneously in both channels. Since the rotational time (0.17 ns) of fluorescein was sufficiently short with regard to the fluorescence lifetime (3.9 ns), we can assume that at long times after the laser pulse, all molecules are relaxed so that any mismatch between the intensities from parallel channel and perpendicular channel was due to different detection sensitivity of the two channels. So the G-factor was obtained by tail matching between the parallel and perpendicular polarized decays of fluorescein.

The rotational correlation times were determined by fitting the data to multi-exponential decays. Figure 4.4 showed the anisotropy decays for free KIF3A/B-GFP motors in solution, EGFP control and fluorescein control. The anisotropy of KIF3A/B-GFP was fit by a bi-exponential decay, yielding a rotational time constant of 0.36 ns for an initial small fast component and a rotational time constant of 30.4 ns for a major slow component (Table 4.1). The decays for EGFP and fluorescein controls were fit to single exponential decays, giving rotational times of 16.8 ns and 0.17 ns, respectively. This huge difference in rotational time between the two fluorophores was consistent with their molecular weights (EGFP: $\sim 29000$ g / mol; Fluorecein: 332 g / mol). For a spherical protein, the rotational time is approximately proportional to its molecular weight (Perrin, 1936). Thus, the molecular weight of KIF3A/B-GFP ($\sim 220$ kD) was too large to account for the 30.4 ns rotational time. On the contrary, because each chain of KIF3A/B had one EGFP molecule fused, this rotational time was more consistent with the rotation of a dimeric EGFP regarding the rotational time of monomeric EGFP (16.8 ns). The free rotation of the EGFP dimer in KIF3A/B-GFP suggests that between the EGFP and the
global motor domain there is a long flexible tether that enables minimum interference with KIF3A/B mobility.

Figure 4.4 Time-resolved fluorescence anisotropy decays for EGFP (black curve in A, curve 2) and EGFP-tagged KIF3A/B motor (red curve in A, curve 1) and for fluorescein control (B).
Table 4.1 Amplitudes and time constants obtained from multi-exponential fit of anisotropy decays for free and bound KIF3A/B-GFP

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Component#1</th>
<th>Component#2</th>
<th>Component#3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_1$</td>
<td>$\tau_1$</td>
<td>$A_2$</td>
</tr>
<tr>
<td>EGFP</td>
<td>0.325</td>
<td>16.8 ns</td>
<td></td>
</tr>
<tr>
<td>Free KIF3A/B-GFP</td>
<td>0.021</td>
<td>0.36 ns</td>
<td>0.318</td>
</tr>
<tr>
<td>0.25µM KIF3A/B-GFP + 10 µM microtubules</td>
<td>0.035</td>
<td>0.85 ns</td>
<td>0.319</td>
</tr>
<tr>
<td>Microtubule-bound KIF3A/B-GFP in the presence of AMP-PNP</td>
<td>0.065</td>
<td>0.71 ns</td>
<td>0.278</td>
</tr>
</tbody>
</table>

When KIF3A/B-GFP motors were bound to microtubules in the presence of ATP, irrespective of the microtubule concentration, the anisotropy decayed in a style similar to free KIF3A/B-GFP motors, with a short fast component followed by a large slow component (Figure 4.5). Because of the large size of free KIF3A/B-GFP motor and KIF3A/B-GFP-microtubule complex, the overall rotational diffusion of the entire molecule was too slow to be resolved within the excited state lifetime of EGFP (2.6 ns). An attempt to fit the fluorescence anisotropy of microtubule-bound KIF3A/B-GFP to a three-component multi-exponential decay function resulted in an infinite time constant for the slowest component that might contain the information about rotational motion of motors bound to microtubules (Table 4.1). That infinitely slow component explains why the anisotropy decay of a mixed sample of free and microtubule-bound KIF3A/B-GFP motors was independent of the microtubule concentration (i.e. the ratio of free motors to bound motors). Therefore it is not practical to use time-resolved fluorescence anisotropy to investigate the affinity of motor-microtubule binding.
Figure 4.5 Time-resolved fluorescence anisotropy decays for microtubule-bound KIF3A/B-GFP motors in the presence of 2mM ATP.

One interesting result was when KIF3A/B-GFP motors were bound to microtubules in the presence of AMP-PNP, the initial fast component was more significant and the slow component corresponding to the segmental mobility of EGFP dimer inside the KIF3A/B-GFP was faster with respect to microtubule-bound motors in the presence of ATP (Figure 4.6 and Table 4.1). It seemed that this result was relevant to the irregular fluorescence lifetime of microtubule-bound motors in the presence of AMP-PNP that we observed before. In section 4.3.1.1, we argued that the fast component in the fluorescence lifetime decay was possibly due to less constraint on the chromophore of
EGFP in the partially denatured KIF3A/B-GFP. If this argument is correct, then we should expect faster segmental mobility of EGFP due to enhanced flexibility (less constraint). This is exactly what we saw, as shown in Figure 4.6 and Table 4.1.

Figure 4.6 Anisotropy decays of microtubule-bound KIF3A/B-GFP at the presence of ATP (curve 1, red) and AMP-PNP (curve 2, black).

4.3.2 Fluorescence correlation spectroscopy

To investigate the reaction kinetics of motor-microtubule binding by FCS, we mixed constant concentration of motors (20 nM) with varying concentrations of microtubules (0.1µM – 10 µM). The fluorescence fluctuations from the sample focused in the femtoliter excitation volume was collected and automatically correlated. The measuring time per sample was 30 – 60 s and all samples were measured two or three times.

Figure 4.7 shows the autocorrelation functions for all the KIF3A/B-GFP samples and conventional kinesin (KHCΔtail-GFP) controls. For ease of comparison the autocorrelation functions were normalized to unity. The correlation curves for free
motors (yellow line in figure 4.7) were significantly different from those for motor-microtubule complexes, with the latter shifted to right meaning larger translational diffusion times for motor-microtubule complexes. The curves for EGFP and EGFP-tagged kinesin were almost overlapped, which is not surprising because the molecular weight of the two protein only differed by ~ 7. Assumed that the diffusion constant is approximately proportional to the cube root of mass, the translational diffusion time of kinesin-EGFP would be only 1.9 times as large as that of EGFP, too small to be easily detected by FCS. In contrast significant differences were observed between free motors and motor-microtubule complexes. As the microtubule concentration was increased, the normalized autocorrelation curves shifted incrementally to the right, suggesting the amplitude of the slow component due to microtubule binding was getting larger and larger. Again, the motor-microtubule complex in the presence of AMP-PNP behaved oddly. Its autocorrelation function (black line) fell directly between those for motors mixed with 0.1 µM (blue line) and 0.3 µM microtubules (pink line). Since the motor-microtubule complex with AMP-PNP was pre-centrifuged, there should not have been any free motors left in this sample unless for some reason (possibly due to laser damage), AMP-PNP could no longer hold motors on microtubules more tightly than ATP and a portion of the motors detached from the microtubules. Additionally, as discussed in section 4.3.1.1, some nonradiative processes may compete for fluorescence in the AMP-PNP sample, which may potentially explain the artifact of the AMP-PNP sample measured in FCS.
Figure 4.7  Normalized autocorrelation curves of constant concentration of EGFP-tagged kinesin motors bound to varying concentrations of microtubules. (A) KIF3A/B-EGFP and (B) KHCΔtail-GFP.

The correlation curves of free KIF3A/B-GFP and KHCΔtail-GFP were fit to Equation 4.8 with a single component (Figure 4.8A and Figure 4.9A), yielding diffusion
times of 1.2 ms and 1.3 ms, respectively. For obtaining the diffusion time of a pure motor-microtubule complex, we chose the sample containing 10 μM of microtubule because it had the highest possibility of having all motors in the bound state and the sample with AMP-PNP failed. The data were fit similarly to the case of free motors and the translational diffusion time were determined to be 4.9 ms for KIF3A/B-GFP and 5.0 ms for KHCΔtail-GFP (Figure 4.8A and Figure 4.9A).

To quantify the fractions of motors in the free state versus in the microtubule-bound state, the autocorrelation functions at varying microtubule concentrations were analyzed in terms of two diffusing species, with a fast component representing free motor specie and a slow component representing the motor-microtubule complex. The data were fit to Equation 4.9 with two components (Figure 4.8B and Figure 4.9B). The diffusion times for motors in the free state and in the bound state were fixed as the values determined by the single-component fitting. The relative amplitude of the fast component and the slow component represented the fractions of motors in the free and bound state, respectively.
Figure 4.8 (A) Autocorrelation curves of KIF3A/B-GFP motor (yellow dot) and motor-microtubule complex (purple dot). The yellow and purple lines represent one-component fit to Equation 4.8 for free and bound KIF3A/B-GFP, respectively. (B) Autocorrelation curves of mixtures of KIF3A/B-GFP and microtubule were two-component fit to Equation 4.9 to obtain the fraction of KIF3A/B-GFP motors in bound states (bound to microtubules).
Figure 4.9 (A) Autocorrelation curves of KHCΔtail-GFP motor (yellow dot) and motor-microtubule complex (purple dot). The yellow and purple lines represent one-component fit to Equation 4.8 for free and bound KHCΔtail-GFP, respectively. (B) Autocorrelation curves of mixtures of KHCΔtail-GFP and microtubule were two-component fit to Equation 4.9 to obtain the fraction of KHCΔtail-GFP motors in the bound state.
To determine the equilibrium dissociation constant ($K_D$), the fraction of motors in the microtubule-bound state was plotted as a function of microtubule concentration for both KIF3A/B-GFP and KHC\textDelta tail-GFP (Figure 4.10).

\[
\text{KIN} + \text{MT} \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} \text{KIN-MT}
\]

For the motor microtubule system above, the dissociation constant can be described as:

\[
K_D = \frac{k_{off}}{k_{on}} = \frac{[\text{KIN}_{\text{free}}] \times [\text{MT}_{\text{free}}]}{[\text{KIN} - \text{MT}]} = \frac{1 - y}{y} \times [\text{MT}_{\text{free}}]
\]

(Equation 4.10)

Since in our system the concentration of total microtubule (0.1 ~ 10 µM) was much higher than that of kinesin motors (20nM), we can assume that after motor binding the amount of free microtubules in the reaction was almost equal to the total microtubule concentration. So,

\[
K_D = \frac{1 - y}{y} \times [\text{MT}_{\text{total}}]
\]

(Equation 4.11)

Rearrange,

\[
y = \frac{[\text{MT}_{\text{total}}]}{K_D + [\text{MT}_{\text{total}}]}
\]

(Equation 4.12)

where $y$ is the fraction of motors in the microtubule bound state.

The motor-microtubule interaction data in Figure 4.10 were fit to Equation 4.12, giving equilibrium dissociation constants of 0.72 µM for KIF3A/B-GFP and 0.28 µM for KHC\textDelta tail-GFP. The fit to the KHC\textDelta tail-GFP data was not as good as that of the KIF3A/B-GFP data, but the value of the dissociation constant determined for KHC\textDelta tail-GFP was in the range of what other people reported using microtubule pelleting assay...
The reliability of the data can be improved by doing more experiments on samples around the equilibrium point and repeating each sample measurement for a few more times. The existence of a few aggregated motors in our samples might affect the data quality. We had to reduce the signal collecting time in order to avoid huge fluorescence spikes caused by diffusion of protein aggregates. Hence, in the future we need to explore a better FCS buffer system for motors and microtubules to reduce aggregation.

![Figure 4.10](image-url) The fractions of motors in the microtubule-bound state determined by FCS were plotted as a function of microtubule concentration. The data were fit to hyperbolas (Equation 4.12), yielding equilibrium dissociation constants of 0.72 µM for KIF3A/B-GFP and 0.28 µM for KHCΔtail-GFP.

The dissociation constants data showed that the microtubule binding affinity of KIF3A/B was lower than that conventional kinesin. Because the affinity is a ratio of the on-rate to the off-rate for each motor type, the on-rate ($k_{on}$) for microtubule binding can be calculated by dividing the off-rate ($k_{off}$) by $K_D$. The off-rate of KIF3A/B-GFP and
KHCΔtail-GFP had been determined as 0.30 s\(^{-1}\) and 0.25 s\(^{-1}\) using TIRF microscopy in chapter 3. Hence, the on-rate of KIF3A/B was calculated to be 0.42 µM\(^{-1}\)s\(^{-1}\) KIF3A/B, which was half of the on-rate of 0.89 µM\(^{-1}\)s\(^{-1}\) for conventional kinesin. This result was consistent with our finding in single molecule fluorescence assays in chapter 3 that KIF3A/B had a lower on-rate than conventional kinesin.

4.4 CONCLUSIONS

In this chapter, the dynamic and kinetic interaction of motors with microtubules was investigated on a fast time scale by time-resolved fluorescence and on a slow time scale by fluorescence correlation spectroscopy.

The measurement of anisotropy decay as a function of time did reveal structural flexibility between KIF3A/B’s tail domains and head domains although it didn’t provide information on the microtubule binding affinity for KIF3A/B motors. The excited state fluorescence lifetime of EGFP did not change upon joining it to motors or upon attaching these motors to microtubules. That result indicates the β barrel enclosing the chromophore is highly preserved during the interaction, setting a nice reference point for fluorescence as measured in anisotropy and FCS. We were able to measure the rapid rotation of EGFP dimers when they were fused to kinesin motors, suggesting that between the EGFP and the motor domain there was a long flexible tether that enabled minimum interference with the KIF3A/B mobility. However, compared to the fast excited state fluorescence decay, the size of KIF3A/B-GFP motors and KIF3A/B-GFP-microtubule complexes are so large that the overall rotational diffusion of the entire molecules is too slow to be resolved.
Using FCS, we have successfully developed a novel method to measure the interaction kinetics of motor-microtubule binding. The observed autocorrelation functions were very sensitive to the relative fractions of motors in the free state versus in the microtubule-bound state. The equilibrium dissociation constants were determined to be 0.72 µM for Kinesin-2 and 0.28 µM for conventional kinesin. In the future, we will apply the same FCS experiment on EGFP-tagged KIF3A/A and KIF3B/B homodimers to characterize the kinetics of each head of KIF3A/B during microtubule binding.
Chapter 5  MONTE CARLO SIMULATION OF KINESIN STEPPING MODEL

5.1 INTRODUCTION

In chapter 3, we showed that single kinesin molecules could take multiple steps (326 steps for conventional kinesin and 83 steps for Kinesin-2) along a microtubule prior to dissociation. To avoid premature detachment for this long processivity, the two heads of a dimeric kinesin must coordinate in a mechanism such that the rear head does not detach until the leading head binds to the next binding site. It’s known that within the 8 nm step, one motor hydrolyzes one ATP and the ATP hydrolysis cycle is tightly coupled to the mechanical stepping cycle. There’s no consensus for the precise sequence of biochemical transitions in the kinesin chemomechanical cycle, and there are a number of competing theories for the precise mechanism underlying the coordination of the two heads of kinesin. Some features of the kinetic cycle are widely accepted while some others are still being debated.

In this chapter, we focused on one of these debates regarding how the rear head detaches upon the binding of the front head: does it detach spontaneously due to the intramolecular strain generated by the front head, or because of the conformational change induced by ATP binding? We incorporated our experimental motility data collected in previous chapters together with kinetic data from the literature into the proposed kinetic schemes for the kinesin chemomechanical cycle. These cycles were computationally analyzed by Monte Carlo simulations, a method of randomly generating values for uncertain variables to yield model data. These models involve a number of rate constants, and standard analytical or computational approaches for calculating
overall cycle rates or head detachment rates are insufficient when modeling single molecule behavior.

The basic idea of using Monte Carlo simulation to model a kinetic reaction is comparing a random number with the probability distributions of transition. At each state of the kinetic system with multiple states, the object has the option of switching to the next state or traveling back to the previous state. The transition between two states is governed by a probability distribution, which is related to the kinetic constants. A random number is chosen, and the dwell time of each transition is calculated based on the random number and the probability distribution. The object will move to the state with the smallest dwell time. This process is allowed to continue for many times until the simulation ends.

By varying the kinetics parameters of the kinesin hydrolysis cycle, Monte Carlo simulations were used to test kinesin models, interpret experimental results and make experimental predictions to drive future experiments.

5.2 MODELS FOR KINESIN STEPPING MECHANISM

Though there is tremendous amount of work on the mechanism of kinesin’s stepping, the structural and chemical transitions in the chemomechanical cycle have not been completely defined. Several different models have been proposed with some aspects broadly agreed upon and some aspects divergent (Cross et al., 2000; Hancock and Howard, 1999; Rice et al., 1999; Schnitzer et al., 2000; Vale and Milligan, 2000). One of the big uncertainties concerns the release of the rear head from the microtubule. It’s established that front head attaches before the rear head detaches. But it’s not clear
whether the intramolecular strain produced by the binding of the front head is enough to pull the rear head off (Cross et al., 2000; Hancock and Howard, 1999), or whether the binding of ATP to the front head is also required (Rice et al., 1999; Vale and Milligan, 2000). The kinetic model presented here combined these two controversial pathways, Spontaneous Detachment (SD) and ATP Induced Detachment (AID) (Figure 5.1).

Figure 5.1 The mechnochemical cycles of kinesin with different mechanisms of how the microtubule-bound head detaches. The left upper branch shows the mechanism of Spontaneous Detachment (SD) in which the rear head detaches as an automatic response to the strain generated by the front head binding. The right upper branch shows the mechanism of ATP Induced Detachment (AID) where the rear head will not detach until the ATP binds to the front bound head. The state of the motors in each conformation is labeled from 1 to 9. State 1 to state 4 correspond to one head and state 5 to state 8 are for the other. State 9 indicates that the motor dissociates off the microtubule. For a homodimeric kinesin such as conventional
kinesin the stepping kinetics for the two heads are identical but for a heterodimeric kinesin such as kinesin-2, they are different.

In state 1, the front head releases its ADP rapidly right after binding, resulted in a tighter binding. In state 2 of the SD pathway, strain generated from the front head binding stimulates the detachment of the rear head and then in state 3, ATP binds to the front attached head. In state 2 of the AID pathway, ATP binds when two heads are in bound states and then in state 3 the additional strain from the ATP binding accelerates the release of the rear head. After these states, the two models share a common pathway with ATP hydrolysis in the attached head in state 4 followed by rapid binding of the front head and release of ADP which is the start of kinesin’s next step. In homodimeric kinesins, the stepping cycles of the two heads are identical. However, in heterodimeric kinesins the kinetic rates for the two heads may be different and then there may be 8 different states in the chemomechnical cycle with 1 to 4 corresponding to one head and 5 to 8 to the other. It’s known that kinesin has weaker affinity to microtubule when its nucleotide state is in the ADP state or ADP•P; state compared to no nucleotide state or ATP state (Hancock and Howard, 1999; Vale et al., 1996). Based on this, we inferred that kinesin dissociates from microtubules only at state 4. This is one of the key steps in formulating a kinesin model.

5.3 MONTE CARLO SIMULATION

5.3.1 Algorithm

In our model, a heterodimeric kinesin existed in eight different states in one cycle with both heads completing a step. For easier demonstration of how the modeling worked, a two-state system was illustrated.
\[
A \xrightarrow{k_1} B \\
\xrightarrow{k_{-1}} B
\]

\[
\frac{dB}{dt} = -\frac{dA}{dt} = \frac{d(A_0 \exp(-k_1 t))}{dt} = A_0 k_1 \exp(-k_1 t)
\]  
(Equation 5.1)

Hence from time 0 to t, the probability of motors making a transition from state A to state B is equal to \( \int_0^t k_1 \exp(-k_1 t) dt = 1 - \exp(-k_1 t) \).  
(Equation 5.2)

If for a randomly generated number \( x \) (between 0 and 1), \( 1 - \exp(-k_1 t) = x \), then \( t = \frac{1}{k_1} \ln \left( \frac{1}{1 - x} \right) \),  
(Equation 5.3)

meaning the elapsed time required for \((100x)\%\) of motors having the transition from A to B done, or in other words the dwell time of transition.

In our system with eight states, the motor can transit in one of three directions: forward along the microtubule, backward along the microtubule or off the microtubule. Rate constants for each of these transitions were input into the Equation 5.3 and the dwell time of a motor transition in each of the three directions was calculated. The motor transited in the direction corresponding to the smallest dwell time. The dwell time and the state number for each transition were recorded. The simulation continued for a given time or until the motor detached, governed by the off rate constant. For the given rate constants, the simulation can yield the time for each transition and hence the dwell time for each step that the motor made, from which the run length and dissociation time before motors detached can be determined. The program was written in MATLAB by undergraduate students James Dizikes, Jeffrey T. Sharps and Jeffrey P. Hafner.
5.3.2 Model parameters and assumptions

The run length data from the single molecule fluorescence assays in chapter 3 were incorporated into the kinetic model as the probability of detachment for each step taken by the motor, \( P_{\text{off}} / \text{step} = 1 - e^{-d/a} \), where \( d \) is equal to 8 nm and \( a \) is the mean run length. The probability that the motor dissociates from the microtubule for a given step is the ratio of rate constants of the front head attaching (\( k_{\text{attach}} \)) and the rear bound head dissociation from state 4 or 8 to state 9 (\( k_{\text{diss}} \)). The \( k_{\text{diss}} \) haven’t been measured, but a good estimation of that is the rate of one-headed kinesin detaching from microtubule in ADP•P\(_i\) state, which is 3 s\(^{-1}\) (Hancock and Howard, 1999). Then based on the dissociation rate and the run length, the \( k_{\text{attach}} \) can be calculated as one of the inputs into the model. Taken from literature, the ATP on-rate and ATP hydrolysis rate was set to 2 µM\(^{-1}\)·s\(^{-1}\) and 200 s\(^{-1}\), respectively (Ma and Taylor, 1995). We measured the overall stepping rates of motors in both single molecule fluorescence assays and microtubule gliding assays and these rates can be used to estimate the detachment rate of the rear head, \( k_{\text{detach}} \). The inverse of \( k_{\text{detach}} \) was equal to the inverse of the overall stepping rate subtracted by the inverse of each of the other rate constants. Most of the reverse rate constants were estimated to be 100-fold less than the forward ones. The reverse rate of ATP unbinding (from state 3 to 2 or state 7 to 6 in SD model and state 2 to 1 or state 6 to 5 in AID model) was set to 40 s\(^{-1}\) so that the ATP concentration at half maximal velocity was equal to 54 µM which was determined from the experimental data of KHCΔtail-GFP (Figure 5.2B).
Table 5.1 Kinetic parameters used as inputs for the simulation of conventional kinesin’s stepping. Parameters were calculated from the motility data of KHCΔtail-GFP and some were taken from literature.

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>State number (SD model)</th>
<th>State number (AID model)</th>
<th>Kinetic rate forward (s⁻¹)</th>
<th>Kinetic rate reverse (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detach</td>
<td>state 1 to 2 state 5 to 6</td>
<td>state 2 to 3 state 6 to 7</td>
<td>210</td>
<td>2.1</td>
</tr>
<tr>
<td>ATP bind</td>
<td>state 2 to 3 state 6 to 7</td>
<td>state 1 to 2 state 5 to 6</td>
<td>2*ATP concentration (µM)</td>
<td>40</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>state 3 to 4 state 7 to 8</td>
<td>state 3 to 4 state 7 to 8</td>
<td>200</td>
<td>2</td>
</tr>
<tr>
<td>Attach</td>
<td>state 4 to 5 state 8 to 1</td>
<td>state 4 to 5 state 8 to 1</td>
<td>1240</td>
<td>12.4</td>
</tr>
<tr>
<td>Both heads dissociate</td>
<td>state 4 to 9 state 8 to 9</td>
<td>state 4 to 9 state 8 to 9</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

5.3.3 Conventional kinesin’s processivity at varying ATP concentrations measured by single molecule fluorescence assays

The SD model and AID model differ in when the ATP molecule binds. They may perform differently when ATP binding is the rate-limiting step at low ATP concentration. Motivated by this, we investigated the processivity of KHCΔtail-GFP across a wide range of ATP concentrations (1µM, 10 µM, 100 µM and 2000 µM) using the same TIRF technique as in chapter 3. The data were shown in Table 5.2 and Figure 5.2.

As discussed in chapter 3, the observed run length and dissociation rate measured by TIRF microscopy should be corrected for photobleaching, especially for highly processive motors under conditions (low ATP) that motors move very slowly and have long residence time. But since each kinesin molecule has two EGFP fluorophores attached, the competition between photobleaching and motor detachment is no longer a simple single exponential decay process, but rather a joint distributed density function which predicts the average photobleaching time of double-labeled motors will be 1.5 longer than that of single-labeled motors (Lakamper et al., 2003). The photobleaching rate was measured to be 0.039 s⁻¹ in chapter 3 and it should be adjusted to 0.026 s⁻¹ for
double-labeled motors. Following Equation 3.3, the run length and dissociate rate can be corrected (Table 5.2). Even after correction for photobleaching, the processivity of KHCΔtail-GFP decreased as ATP concentration reduced.

**Table 5.2** Motility of KHCΔtail-GFP measured at different ATP concentrations in single molecule fluorescence assays.

<table>
<thead>
<tr>
<th>ATP Concentration (µM)</th>
<th>Runlength (µm)</th>
<th>Off Rate (s⁻¹)</th>
<th>Velocity (nm/s ± S.D.)</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Corrected</td>
<td>Observed</td>
<td>Corrected</td>
</tr>
<tr>
<td>2000</td>
<td>2.93</td>
<td>3.27</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>100</td>
<td>2.84</td>
<td>3.33</td>
<td>0.18</td>
<td>0.15</td>
</tr>
<tr>
<td>10</td>
<td>1.26</td>
<td>1.88</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>0.61</td>
<td>1.06</td>
<td>0.06</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Figure 5.2** Motility of KHCΔtail-GFP at varying ATP concentrations measured in single molecule fluorescence assays. (A) Run length plotted as a function ATP concentration. (B) Velocity of motors
plotted as a function of ATP concentration. The velocity data were fit to the Michaelis-Menten equation, yielding 54 µM to be the ATP concentration for half maximal velocity.

### 5.3.4 Monte Carlo Simulation of conventional kinesin’s processivity at varying ATP concentrations

We employed Monte Carlo computation to simulate the above single molecule fluorescence experiments. Simulations of both AID model and SD model were run using the inputs from Table 5.1 across the same range of ATP concentrations as the above TIRF experiments. A single motor simulation consisted of repeated hydrolysis and stepping cycles until the motor detached from the microtubule. In each model simulation, five hundred independent motors were simulated to generate statistics for stepping speeds and run lengths (Figure 5.3). The outputs were summarized in Table 5.3 for AID model and in Table 5.4 for SD model.

**Table 5.3 Simulated motility of KHCΔtail-GFP following ATP induced detachment (AID) model**

<table>
<thead>
<tr>
<th>ATP Concentration (µM)</th>
<th>Runlength (µm)</th>
<th>Off Rate (s⁻¹)</th>
<th>Velocity (nm/s ± S.D.)</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>3.26</td>
<td>0.22</td>
<td>729</td>
<td>N = 500</td>
</tr>
<tr>
<td>100</td>
<td>3.14</td>
<td>0.15</td>
<td>480</td>
<td>N = 500</td>
</tr>
<tr>
<td>10</td>
<td>1.91</td>
<td>0.06</td>
<td>113</td>
<td>N = 500</td>
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<tr>
<td>1</td>
<td>0.39</td>
<td>0.03</td>
<td>13</td>
<td>N = 500</td>
</tr>
</tbody>
</table>

**Table 5.4 Simulated motility of KHCΔtail-GFP following spontaneous detachment (SD) model**

<table>
<thead>
<tr>
<th>ATP Concentration (µM)</th>
<th>Runlength (µm)</th>
<th>Off Rate (s⁻¹)</th>
<th>Velocity (nm/s ± S.D.)</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>3.22</td>
<td>0.23</td>
<td>730</td>
<td>N = 500</td>
</tr>
<tr>
<td>100</td>
<td>3.30</td>
<td>0.14</td>
<td>479</td>
<td>N = 500</td>
</tr>
<tr>
<td>10</td>
<td>3.07</td>
<td>0.04</td>
<td>112</td>
<td>N = 500</td>
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<tr>
<td>1</td>
<td>2.96</td>
<td>0.004</td>
<td>13</td>
<td>N = 500</td>
</tr>
</tbody>
</table>

The AID model predicted a dramatic decrease of processivity at limiting ATP concentration while in the SD model there were no significant changes of processivity as
ATP level reduced. One plausible explanation of reduced processivity in AID model is that at low ATP concentration motors will accumulate in state 1 or 5 waiting for ATP binding and for a highly processive motor the $k_{\text{attach}}$ is very fast such that there’s a significant reverse rate back to state 4 or 8 where dissociation of motors from microtubule occurs. In the SD model, at low ATP level motors stay in state 2 or 6 with front head bound and rear head free and because releasing the rear head from the microtubule is usually the rate limiting step at saturated ATP concentration, the $k_{\text{detach}}$ is so small that the reverse stepping to state 1 or 5 and then to state 8 or 4 is impossible, indicating the probability of motors detaching from the microtubule will not be increased.

To test this explanation, the reverse rates from state 5 to 4 and 1 to 8 were set to zero. We found that the dependence of processivity on ATP concentration vanished and the processivity maintained at a constant level across all ATP concentrations after the reverse stepping of front head detachment was removed.

The simulation data were plotted and compared with the experimental data in Figure 5.4. It was clearly shown that as ATP concentration varied, the processivity and dwell time from the experimental data followed similar patterns as the AID simulation data, rather than the SD data. Therefore, our experimental data supported the AID model and excluded the SD model.
Figure 5.3 Histograms of run length distributions of KHCΔtail-GFP at varying ATP concentrations from TIRF experiments (left panel), simulations of AID model (center panel) and simulations of SD model (right panel).
Figure 5.4 Correlation of experimental KHCΔtail-GFP data (red square and line) with Monte Carlo simulation data of AID model (blue dot and line) and SD model (purple triangle and line). (A) Run length
plotted as a function ATP concentration. (B) Dwell time of motors on microtubule plotted as a function of ATP concentration. (C) Velocity of motors plotted as a function of ATP concentration.

There were 9 different kinetic parameters in the simulation of conventional kinesin (Table 5.1 and Figure 5.1). The ATP on-rate ($k_{\text{on}}^{\text{ATP}}$) and ATP hydrolysis rate ($k_{\text{hydrolysis}}$) were taken from literature and the values are reliable. When these two parameters varied in a moderate range, there were no significant effects on simulation results. The relationship between $k_{\text{on}}^{\text{ATP}}$, $k_{\text{off}}^{\text{ATP}}$ (ATP off-rate) and $k_{\text{hydrolysis}}$ is mostly determined by the ATP concentration at half maximal velocity ($K_m$), which was measured to be 54 µM (Figure 5.2B). Then $k_{\text{off}}^{\text{ATP}}$ can be chosen so that the $K_m$ predicted by the model is consistent with the experimental value. The detachment rate of the rear head ($k_{\text{detach}}$) was estimated from velocity measurements. The attachment rate of front head ($k_{\text{attach}}$) was derived from the run length data and it was dependent on the dissociation rate, $k_{\text{diss}}$, which is one of the uncertain parameters. Another uncertainty is $k_{-\text{attach}}$, the reverse rate of front head attaching (from state 5 to 4 in Figure 5.1). Sensitivity analysis was performed for these two parameters in both AID simulation and SD simulation. As $k_{\text{diss}}$ and $k_{-\text{attach}}$ varied in two orders of magnitude, there were no significant changes in the simulated run lengths and velocities for both the AID model (shown in Figure 5.5 and Figure 5.6) and the SD model (data not shown). An exceptional case was when the $k_{-\text{attach}}$ was chosen as small as 1.24 s$^{-1}$ in the AID model, the predicted run lengths dropped more slowly than those with larger $k_{-\text{attach}}$ as ATP concentration reduced (Figure 5.6A).
Figure 5.5 Monte Carlo simulation data of AID model for KHCΔtail-GFP at varying dissociation rates ($k_{\text{diss}}$). (A) Run Length. (B) Velocity.
5.3.5 Monte Carlo Simulation of Kinesin-2’s processivity at varying ATP concentrations

Similarly, the processivity of Kinesin-2 at varying ATP concentrations was simulated for both the recent preps of EGFP-tagged KIF3 motors with point mutations

Figure 5.6 Monte Carlo simulation data of AID model for KHCA\Delta tail-GFP at varying reverse rates of front head attaching ($k_{\text{attach}}$). (A) Run Length. (B) Velocity.
fixed and for the old preps of full length KIF3 motors which contained couple of point mutations (See discussion of Chapter 3).

5.3.5.1 EGFP-tagged KIF3 motors with point mutations fixed

The processivity and moving speeds of wild-type KIF3A/B-GFP heterodimer and chimaeric KIF3A/A-GFP and KIF3B/B-GFP homodimers were measured by single molecule fluorescence assays in chapter 3. The KIF3A/A-GFP and KIF3B/B-GFP were simulated exactly the same as conventional kinesin with two heads having identical kinetic cycles. But for heterodimeric KIF3A/B-GFP, we assumed that when the two heads were joined together each of them would retain the same stepping rate and front head attachment rate ($k_{attach}$) as in the context of homodimers. And those values were used as the inputs into the simulations. For both AID model and SD model, the predicted run length of KIF3A/B at saturated ATP concentration agreed extremely well with our experimental measurement (Figure 5.7A and Figure 5.7B), roughly the average of the run lengths of the two heads. Since we didn’t have the data at low ATP concentrations, models couldn’t be tested here. For all three constructs, the AID model predicted the same processivity dependence of ATP concentration as for conventional kinesin, lower processivity at low ATP level.
Figure 5.7 Monte Carlo simulation data of AID model (A) and SD model (B) for KIF3A/B-GFP (purple), KIF3A/A-GFP (red) and KIF3B/B-GFP (blue).

5.3.5.2 Full length KIF3 motors with point mutations

For mutant KIF3A/B, KIF3A/A and KIF3B/B that contained some random point mutations, we only had the velocity data from microtubule gliding assays. The front head attachment rates \((k_{attach})\) were estimated from values for EGFP-tagged KIF3 motors. This may create a risk for the KIF3A/A since in the KIF3A sequence there was a point
mutation occurred in the microtubule-binding site, which may decrease the affinity for microtubules. Both models demonstrated that to ensure the overall stepping rate of KIF3A/B at saturated ATP concentration, the slow A head had to be accelerated at least 3 times by the fast B head, consistent with our previous predictions in chapter 2.

The velocity of mutant KIF3 motors were measured in microtubule gliding assays across a wide range of ATP concentrations (1µM, 3 µM, 10 µM, 30 µM, 100 µM, 300 µM and 1000 µM). Both the experimental data and the simulation data for AID model and SD model were plotted in Figure 5.8 and fit to the Michaelis-Menten equation and the ATP concentrations at half maximal velocity ($K_m$) were listed in Table 5.5. For mutant KIF3A/A, the $K_m$ resulted from the SD simulation was far less than the experimental value no matter what kinetics parameters were chosen for the simulation while the simulation data from the AID model were in better agreement with the experimental data, providing another evidence for supporting the AID model.
Figure 5.8  Correlation of experimental velocity data of mutant KIF3 motors (red triangle) with Monte Carlo simulation data of AID model (blue dot) and SD model (purple square).  (A) Velocity of mutant KIF3A/A plotted as a function of ATP concentration.  (B) Velocity of mutant KIF3B/B plotted as a function of ATP concentration.  (C) Velocity of mutant KIF3A/B plotted as a function of ATP concentration.  The data were fit to the Michaelis-Menten equation (red, blue, purple lines).
Table 5.5 ATP concentrations at half maximal velocity ($K_m$) of mutant KIF3 motors determined from microtubule gliding assays and from AID and SD simulations

<table>
<thead>
<tr>
<th></th>
<th>Mutant KIF3A/A</th>
<th>Mutant KIF3B/B</th>
<th>Mutant KIF3A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>19 µM</td>
<td>14 µM</td>
<td>25 µM</td>
</tr>
<tr>
<td>AID Simulation</td>
<td>21 µM</td>
<td>37 µM</td>
<td>28 µM</td>
</tr>
<tr>
<td>SD Simulation</td>
<td>3 µM</td>
<td>31 µM</td>
<td>14 µM</td>
</tr>
</tbody>
</table>

Figure 5.9 showed the simulated run length data for mutant KIF3 motors at varying ATP concentrations. Lower processivity were expected at low ATP concentrations in AID model but not SD model, similar to conventional kinesin.

![Figure A.](image1)

![Figure B.](image2)

**Figure 5.9** Monte Carlo simulation data of AID model (A) and SD model (B) for mutant KIF3A/B (purple), KIF3A/A (red) and KIF3B/B (blue).
5.4 CONCLUSIONS AND DISCUSSIONS

In this chapter, Monte Carlo method was used to simulate two competing models for kinesin’s chemomechanical cycle. Our experimental data of kinesin’s processivity at varying ATP concentrations supported the prediction from the ATP Induced Detachment (AID) model that run length decreased substantially at low ATP concentrations.

Our experimental data agreed with the work by Schnitzer et al (Schnitzer et al., 2000), who found that at low ATP concentrations and low loads run length of kinesin measured by optical trapping decreased markedly and their data also suggested that the ATP dependence of run length was strongest at zero load. However, both our work and this work conflict with the report of Yajima et al that kinesin run length at low load was independent of ATP concentration in the µM to mM range. Yajima measured run lengths of rat conventional kinesin coupling rhodamine-labeled actin filaments to kinesin motors via a gelsolin linker at kinesin’s C-terminal, enabling the experiments to be conducted by epifluorescence microscopy without significant photobleaching (Yajima et al., 2002).

Photobleaching is a problematic issue in the single molecule TIRF assay, especially at low ATP concentrations when the dwell times of motors on the microtubule were longer. At 1 µM ATP concentration, the dwell time was measured to be 20 s, which was only slightly shorter than the photobleaching decay constant, 38 s, as measured in chapter 3. We may argue that the small run length we observed at this extremely low ATP level was simply due to photobleaching, though we corrected our run length with a bleaching factor and don’t believe this to be the case. At 10 uM ATP concentration, we still observed a remarkable decrease of run length and it could not be completely explained by photobleaching because this time the dwell time was 15 s.
In the future, to make the model testing more valid, motors labeled with fluorophores that can sustain photobleaching during long-term observation will be explored into the single molecule fluorescence measurements of processivity. Kinesin labeled by quantum dots are a good candidate for this experiment.

Another concern was the contaminated ADP level in the sample because I did not include an ATP-regenerating system. If the ADP level in the sample is sufficient to compete with ATP and detach the motor from microtubule at state 2 or 6, the SD modeling needs to be revised due to the additional dissociation path at state 2 or 6, yielding a new prediction similar to the AID model. However, Yajima et al showed that when ADP was combined with an equal molar ratio of ATP, the run length only decreased by 45% (Yajima et al., 2002). Therefore, the tiny amount of ADP contaminants in our system should not bias our results.
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VITA

Yangrong Zhang
205 Hallowell Building, University Park, PA 16801
Mobile: (814) 769-6021, Fax: (814) 863-0490
Email: yyz103@psu.edu

Education
2000 – present            Ph.D. candidate            Department of Bioengineering
                           The Pennsylvania State University
1995 – 2000              B.S.                             Department of Molecular & Cell Biology
                           University of Science and Technology of China

Experience
2000 – present            Research Assistant            Molecular Biomechanics Lab
                           The Pennsylvania State University
2003 – 2004              Teaching Assistant            Department of Bioengineering
                           The Pennsylvania State University

Publication and Manuscripts in Preparation
- **Y. Zhang** and W. O. Hancock, “Processivity of EGFP-tagged KIF3A/B Quantified in Single Molecule fluorescence assay”, *Manuscript in preparation*

Awards & Affiliations
- Member, Biophysical Society, 2005, 2004
- Member, The American Society for Cell Biology, 2003
- Hua Xin Award for Excellence in Undergraduate Study, University of Science & Technology of China, 1997
- Outstanding Student Scholarship, University of Science & Technology of China, 1996
- Third Prize Award for National Contest of Chemistry, 1995