

Literature Review and Project Introduction

MTs and Motor Proteins

Microtubules (MTs) perform an essential role in cellular development and are important for a myriad of cellular functions. Over the past two decades, MTs have been implicated as not only a basic part of the cellular cytoskeleton, giving desired shape and polarity, but also as "highways" for intracellular organelle transport. Particular enzymes collectively known as motor proteins move along these "highways". Microtubule-associated motor proteins function as the driving force for microtubule-dependent motile phenomena, including membrane transport and chromosome movement during meiosis and mitosis. The locomotion of motor proteins is due to hydrolysis of ATP and the subsequent release of ADP and phosphate. The characteristic movement can be described as walking or sliding along the MT.

MTs have a definite shape and polarity, and can be organized in four fashions : 1) a radial array in interphase cells, 2) a bipolar spindle in mitosis and meiosis, 3) a parallel array in polarized epithelial cells, and 4) a linear array in axons. A single microtubule is a hollow cylinder, 25 nm in diameter, and is composed of 13 protofilaments made up of tubulin protein dimers. One tubulin dimer has a molecular mass of 100 kD, and is composed of an α subunit and a β subunit. Each dimer is composed of α and β tubulin, two closely-related proteins, each composed of approximately 450 amino acids. Tubulin dimers assemble into long protofilaments, and interactions between the tubulin dimers maintain the tubular shape of the MT. MTs display an intrinsic polarity, generated by the head-to-tail assembly of alpha and beta tubulin dimers. One end of a microtubule is termed the "plus" end, and the other end is termed the "minus" end. At the plus end, tubulin assembly is 2-3 times faster than at the minus end (Mitchison, 1986). Analysis using anti-alpha tubulin antibodies has determined that the minus end is comprised of alpha tubulin subunits; therefore, the "plus" end is thought to be comprised of beta tubulin subunits (Fan et al., 1996).

Microtubule polarity is important for the cellular organization that promotes proper chromosome segregation. MTs are oriented with the minus end closest to the microtubule-organizing center (MTOC), and the plus end farthest away. The kinetochore MTs are the subset of MTs that attach to the kinetochore as chromosomes migrate to the poles of the future daughter cells. Microtubule motor proteins also play an important role in the movement of chromosomes. Many motor proteins are thought to move along MTs as the chromosomes are pulled apart, while others are found strictly on the kinetochore region, like glue holding the microtubule in place. Proper microtubule assembly and organization are necessary for proper motor protein function. In fact, it is

the intrinsic polarity of a microtubule that guides the motor in its role within the cell. A motor protein can be classified as "plus" end directed, moving towards the plus end of the MT and away from the MTOC, or "minus" end directed, moving towards the MT minus end and towards the MTOC.

In a process known as dynamic instability, MTs undergo alternating phases of growth and rapid shortening (Mitchison and Kirschner, 1984; Walker et al., 1988). There exist two subsets of MTs within a MT population; one shows an overall increase in length while the other shows a decrease in length. A single growing microtubule can suddenly convert to shortening. This transition to shortening from the elongation phase is called a catastrophe, and a subsequent return to the elongation phase is termed a rescue (Walker et al., 1988). Dynamic instability of individual MTs has been studied *in vitro* and *in vivo* using both video-enhanced differential interference contrast (DIC) and fluorescence microscopy (Cassimeris et al., 1988; Cassimeris et al., 1988; Walker et al., 1988). MT assembly and disassembly are believed to be a function of GTP hydrolysis. Tubulin dimers are GTPases that hydrolyze GTP during MT-assembly. There are two sites for GTP binding on tubulin dimers: one exchangeable site (E) on beta tubulin and one non-exchangeable site (N) on alpha tubulin. The rate of polymerization is affected by the amount of GTP-bound tubulin. As GTP-tubulin dimers are incorporated into growing MTs, the GTP is hydrolyzed at the E site and the resulting MT has a tubulin-GDP core. The MT cap or top crown of tubulin dimers remains as GTP-tubulin. This GTP-cap is believed to be important in maintaining MT polymerization. When a MT loses its GTP-cap by hydrolysis or dissociation, it undergoes a catastrophe and rapidly shortens (Walker et al., 1989). In a similar fashion, a rescue can occur when the MT obtains a new GTP-cap, and the MT returns to the elongation phase. Hydrolysis of GTP is thus required for dynamic instability. It is believed that GTP-tubulin can only be found in the MT cap, since to this date, biochemical tests have not been able to locate any unhydrolyzed GTP within the MT core (Erickson-Lamy et al., 1992).

Characteristics of Microtubule Motor Proteins

Two major MT-motor protein families are known, the dynein family and the kinesin family, and members of these two families share a similar characteristic structure. Microtubule motors are composed of three domains: a head, a stalk, and a tail. The head/motor domain may contain one or two large globular "heads." Each head contains an ATP binding site responsible for movement, and a nucleotide-dependent microtubule-binding site. The head domains must interact with the MTs for successful movement. The "stalk" domain forms an α -helical coiled coil and is necessary for dimerization. Lastly, the small, globular "tail" domain is involved in binding the motor to specific cargo, like kinetochore proteins, various vesicles, or other MTs. Each motor has a unique tail to transport particular cargo. Tails that bind MTs contain an ATP-independent MT-binding site. These

proteins are necessary for sliding MTs past one another in formation of the mitotic spindle. Related tails from particular classes of proteins may contain related recognition sites for the binding of a certain cargo (Stewart et al., 1991). The tail regions are not currently well characterized, but are obviously important for cell structure and vesicular traffic within the cell.

The Dynein Family

Axonemal dynein was discovered in 1963, and cytoplasmic dynein was discovered some 25 years later (Gibbons, 1963; Porter and Johnson, 1989). Axonemal dynein is involved in the movement of cilia and flagella where it is important for proper internal structure. An axoneme is an internal bundle composed of two inner MTs with nine surrounding MT-doublets, and axonemal dynein is important for translocating MTs that results in bending of the axoneme. Cytoplasmic dynein was originally referred to as Microtubule Associated Protein One C (MAP 1C), and was identified due to its association with brain MTs. Cytoplasmic dynein is important for retrograde axonal transport, movement towards the cell body of a neuronal cell. This is movement towards the minus-end of the MT; hence cytoplasmic dynein is a minus end-directed motor protein, as are all known dyneins (Asai, 1996). Cytoplasmic dynein is also involved in transport and positioning of the Golgi apparatus in cells, in chromosome segregation, and movement of daughter nuclei at the close of cell division (Asai, 1996; McIntosh and Pfarr, 1991; Skoufias and Scholey, 1993; Vallee, 1990; Walker and Sheetz, 1993).

Dyneins are large motor proteins. One dynein protein is composed of two or three heavy chains, corresponding to the force-generating head domains and stalk domain of kinesin, that are greater than 400 kD (Paschal and Vallee, 1987). Dynein also contains two or three intermediate chains and 4-8 light chains, corresponding to the tail domain of kinesin, that interact with the heavy chains and contribute to the large size of the motor (Vale and Toyoshima, 1988). Currently the function of intermediate and light chains is not well understood, but they may play a role in protein regulation by interacting in some manner with the motor domain.

The Kinesin Family

The second MT-motor protein family is the kinesin superfamily, composed of kinesin and kinesin-like proteins. Kinesin-like proteins can be plus-end or minus-end directed, while kinesin is a plus-end directed motor. Motors in the kinesin superfamily all share a 35-40% sequence homology in their motor domains (approximately 340 amino acids), and exhibit microtubule-stimulated ATPase activity. Kinesin in total is a 360 kD heterotetramer, a smaller protein than dynein. Kinesin consists of two heavy chains and two light chains. Kinesin-like proteins consist of one or two heavy chains and one or two light chains. The heavy chains form a dimer mediated by the formation of an alpha-helical coiled coil in the stalk (Walker and Sheetz, 1993). Again, the function(s) of the light chains is

unclear. It is postulated that the light chains play some role in movement, or regulation, and may interact with the heavy chains.

Kinesin was first identified in the axons of squid, where it is involved in anterograde transport (movement towards the plus end of MTs and away from the neuronal cell body) (Vale et al., 1985). Upon addition of AMP-PNP, a non-hydrolyzable analog of ATP, to squid axoplasm, normally transported organelles became tightly bound to the MTs of the axon. A myriad of kinesin and kinesin-like proteins were subsequently identified in various organisms from yeast to human. The kinesin superfamily of over sixty members is divided into eight smaller, more interrelated subfamilies of kinesin and kinesin-like proteins (Goodson et al., 1994). These subfamilies were defined based on direct experimental identification, amino acid similarities, and functional roles. This classification scheme provides an indication of the many different roles that motor proteins play, including: intracellular transport of membrane-bound organelles, anterograde and retrograde axonal transport, mitotic and meiotic spindle development and organization, spindle pole body and centrosome separation, and transport of synaptic vesicles and mitochondria. The KHC (kinesin heavy chain) subfamily members are all plus-end directed proteins involved in intracellular transport of membrane-bound vesicles and organelles. This subfamily contains the originally-identified squid kinesin that was mentioned above. Members of this family, specifically *Drosophila* kinesin, are characterized in the results section of this paper. The Unc-104 subfamily is named after a plus-end directed motor from *C. elegans* (Hall and Hedgecock, 1991). These plus-end directed proteins may be monomers (having one head) and are implicated in movement of mitochondria and synaptic vesicles. The KRP85/95 subfamily of plus-end directed motors is named for proteins identified in sea urchin (Cole et al., 1993). These proteins play roles in anterograde transport. The C-terminal motor subfamily contains two well-studied proteins, Ncd and Kar3 (Hatsumi and Endow, 1992; Meluh and Rose, 1990). The Ncd protein will be discussed in the experimental part of this paper. All the previously-mentioned subfamilies contain proteins with motors located at the N-terminus. The members of the C-terminal subfamily are involved in mitotic and meiotic spindle function. The bimC subfamily contains plus-end directed motors that are slower-moving proteins. These proteins function in spindle pole and centrosome separation during spindle formation. BimC was isolated from *Aspergillus nidulans*, one of the first KLPs identified (Enos and Morris, 1990). The MKLP1 subfamily contains plus-end directed proteins that associate with the spindle. The mitotic-like-kinesin 1 and other proteins may bind to chromosomes directly at the metaphase plate (Nislow et al., 1992; Vernos et al., 1993). The eighth subfamily, MCAK/KIF2, includes plus-end directed proteins of diverse roles including vesicle transport and cell division. The namesakes were identified in Chinese hamster ovary cells and mouse, respectively (Noda et al., 1995; Wordeman and Mitchison, 1995). Each subfamily contains proteins identified in various organisms. Since 1985, there has been a surge in the discovery of kinesin-like proteins, which led to the creation of the kinesin superfamily tree.

Perhaps there are many more proteins with varied cellular roles still undiscovered. Studies are currently underway to fully characterize the activities and cellular location of each motor protein.

Movement of a MT-motor protein as exemplified by kinesin

Movement of MT-motor proteins depends on cooperative interactions between the two heads of a dimeric protein (Gelles et al., 1988). This process has been well-characterized and can be best-explained by using kinesin as an example. Kinesin moves along a microtubule between regularly-spaced binding sites. This is referred to as the kinesin step size, which is equivalent to the distance between tubulin dimers (8 nm) (Howard, 1995; Svoboda et al., 1993). Kinesin generates movement in a nucleotide-dependent manner. Four nucleotide states exist: a nucleotide free state, an ATP bound state, an ADP + Pi bound state, and an ADP bound state. The cyclic process allows movement via repetitive binding and releasing of the microtubule, coordinated with cycles of nucleotide binding and hydrolysis (Romberg and Vale, 1993). Since movement is nucleotide dependent, kinesin's affinity for the microtubule varies with the nucleotide. In the presence of ATP, kinesin binds weakly to MTs. In an ADP-bound state, kinesin also has a weak affinity for MTs (Hirose et al., 1995), while in the absence of nucleotide kinesin binds tightly to MTs. Under experimental conditions, with AMP-PNP kinesin binds tightly to MTs. MTs stimulate the ATPase rate by increasing the rate of ADP release, the rate-limiting step (Huang and Hackney, 1994). In the absence of MTs, kinesin hydrolyzes ATP at a slower rate, termed the basal rate.

Movement along a MT can be described as a series of steps undertaken by kinesin as it walks along a MT. One head is initially bound to a microtubule, and this head undergoes a conformational change induced by the hydrolysis of ATP. This action brings the second head forward into the correct position at the next binding site on the MT. This all takes place rapidly, as the first head releases the microtubule after the second head has found its new binding site. The attached head rotates, bringing the trailing head into correct position on the microtubule. The ATP-bound state is attached and ATP hydrolysis induces the next rotation. This pattern occurs repetitively as kinesin walks "head over head" along the microtubule, where one head grips the microtubule and the other is free to move to a new binding site. In this manner, one head is always bound to the microtubule, allowing for continuous movement. Electron microscopy and cross-linking experiments have previously indicated that there is one kinesin head binding site per tubulin heterodimer (Kikkawa et al., 1995). A unique feature of kinesin is its ability for processive movement. A single kinesin molecule can move along and stay attached to a MT for prolonged periods of time (Block et al., 1990; Howard et al., 1989). Biochemical analysis has shown that kinesin can hydrolyze approximately 100 molecules of ATP as it moves along a single MT (Hancock and Howard, 1998). These properties are necessary for kinesin's cellular roles, including organelle transport over long distances in a short period of time.

Movement depends on the kinesin motor domain, corresponding to the first 340 amino acids of the N-terminus in a plus-end directed motor or approximately the last 340 C-terminal amino acids of a minus-end directed motor (excluding any C-terminal regulatory domains, e.g. calmodulin binding) (Yang et al., 1989). In a plus-end directed motor, like *Drosophila* kinesin, the bound head directs the unbound head toward the plus end of the microtubule. In a minus-end directed motor, like Ncd, the bound head directs the unbound head towards the minus end. The molecular basis for this directionality is unclear, but may be due to biased weak binding of the motor to MTs (Lockhart and A., 1994). It is interesting to note that both plus-end and minus-end directed motors contain the conserved motor domain, with 40% amino acid identity. This fact recently has shed light on regions other than the motor that might play a role in determining directionality. Recent experiments have studied the “neck” region as a possible “paw” in controlling directionality (Endow and Fletterick, 1998; Endow and Waligora, 1998; Sablin et al., 1998). The neck region is the short region between the head and stalk, and it can be identified by analysis of the motor domain crystal structures, and by amino acid sequence similarity. The neck regions in Ncd and kinesin were found to be similar in sequence, but are comparatively disordered in the crystal structures of both motor domains (Kull et al., 1996). The neck region may allow for a conformational change during ATP hydrolysis that would subsequently result in movement towards one end of the MT (Endow and Fletterick, 1998; Endow and Waligora, 1998; Sablin et al., 1998). For this reason, it is believed that the neck domains of kinesin and Ncd are disordered compared to one another, allowing for movement towards the plus end or minus end, respectively. The neck residues directly adjacent to the conserved motor core, specifically within four residues, are especially critical for Ncd minus-end directed movement, as shown in a recent protein analysis study using Ncd and *Drosophila* kinesin heavy chains (Endow and Waligora, 1998). Again, structural analysis can be combined with this previous study to identify important residues like those involved in formation of characteristic alpha-helical coiled coils (Kull et al., 1996). Electron micrograph reconstructions originally opened the idea for the role of the neck/stalk domain in directionality. These micrographs indicated that the unattached head of dimeric kinesin pointed towards the plus end of the MT and similarly showed that the unattached head of dimeric Ncd pointed towards the minus end (Arnal et al., 1996; Hirose et al., 1996). It is this conformational change and the release of ADP that drives movement of the neck region, also called the lever arm (Cross, 1997). In another study, the head of the minus end protein, Ncd, was attached to the tail region of a plus end directed kinesin, Nkin from *Neurospora crassa* (Henningesen and Schliwa, 1997). Surprisingly, this chimeric motor protein moved towards the plus end of the MT; therefore, the tail may be involved in conferring directionality as well. At this time, exactly what is responsible for directionality is not clear.

Cysteine Modification of MT-Motor Proteins

Chemical modification is a powerful technique for probing functionally important amino acids. Various sulfhydryl-reacting reagents have been used to treat proteins and analyze their functional activities. These different reagents have varying abilities to alkylate or modify cysteines. Additionally, cysteines within the same protein have varying reactivities. Commonly-used sulfhydryl-reacting chemicals include maleimides, iodoacetamides, and ethacrynic acid. One extensively-used sulfhydryl reacting agent is N-ethylmaleimide (NEM). NEM was identified as a powerful antimiotic agent with the ability to alkylate sulfhydryl groups (Means and Feeney, 1971). NEM reacts readily with exposed sulfhydryl groups and has been used to determine the location and the role of these groups in the function of numerous proteins.

Previously, NEM has been shown to inhibit the activity of motor proteins. For example, NEM modification has provided insight into structure-function relationships of myosin. Myosin is the motor protein involved in force generation of muscle, and interacts with actin filaments like kinesin interacts with MTs. NEM reactions with myosin led to the identification of two essential sulfhydryl groups in this protein, identified as SH1 (Cys-707) and SH2 (Cys-697) (Grolig et al., 1996). This is a good example of cysteines with varying reactivities, SH1 being more reactive than SH2. NEM inhibits release of actin by myosin and hinders actin ATPase activity. The two sulfhydryl groups are thought to be involved in the ATP binding sites of myosin and may be involved in nucleotide binding and hydrolysis activities (Meeusen and Cande, 1979).

Kinesin and dynein are also affected by sulfhydryl modification. Effects of modification on motor proteins may be analyzed with a motility assay, a sedimentation assay, and an ATPase assay. In the motility assay, motor-driven microtubule movements are observed via video-enhanced microscopy (VE-DIC). The motility assays are scored for microtubule binding, length of bound MTs, and rate of translocation. The sedimentation assay tests the motor's ability to bind MTs in the presence of ATP or AMP-PNP. ATPase activity assays reveal the effect of modification on a motor's basal and microtubule-stimulated ATPase rates (Huang et al., 1994).

Dynein is significantly more sensitive to sulfhydryl modification by NEM than is kinesin. Cytoplasmic and axonemal dynein are both inhibited by relatively low concentrations of NEM (<100 μM) (Martenson et al., 1995). In a motility assay with dynein and ATP, MTs glide smoothly along the coverslip surface. Modified dynein loses the ability to support MTs in a dose-dependent manner (Martenson et al., 1995). Upon modification, dynein shows a decrease in the amount and rate of microtubule translocation; however, modification results in increased microtubule binding i.e., modified dynein has a strong affinity for those MTs already bound. All microtubule movement is lost with NEM concentrations as low as 10 μM (Martenson et al., 1995). To date, sedimentation assays

have not been performed on dynein, but modified dynein shows a reduced rate of ATPase activity. It is hypothesized that the critical cysteine may be readily accessible in dynein's motor domain, since it appears necessary for movement of MTs and is sensitive to low concentrations of NEM.

In the presence of ATP, kinesin, like dynein, glides MTs unidirectionally across coverslips. However, kinesin treated with NEM loses its ability to bind MTs (Walker et al., 1997). Interestingly, NEM treatment of the kinesin-like protein Ncd blocks microtubule gliding and not attachment, similar to NEM-modified dynein (Walker et al., 1990). Unlike modified dynein, modified kinesin does not show a reduction in rate of microtubule movement (Martenson et al., 1995). The NEM dose required (1-5 mM) to obtain these effects is about one hundred times higher than what is required for similar effects shown with dynein. It is not clear why such high concentrations are needed. In addition to the higher concentrations, kinesin modification also requires a temperature of 37°C, which is thought to unfold the protein and allow NEM to reach relatively inaccessible sites. Treatment of native chick kinesin with [³H]NEM identified seven reactive sites (Walker et al., 1997). When quantified, the time course of labeling showed that the light chains in native chick kinesin are modified before the heavy chains are fully modified. This may indicate that the reactive cysteines in kinesin heavy chain are relatively inaccessible, and that the light chains are labeled first, ultimately leaving other cysteines in the heavy chain accessible to NEM. It is interesting to note that many cysteines in kinesin are conserved across species. This fact could provide evidence the importance of a particular critical cysteine in kinesin proteins.

Modified kinesin is unable to bind MTs in the presence of ATP in a motility assay; however, modified kinesin still binds MTs when forced to by addition of AMP-PNP (Walker et al., 1997). In other words, if ATP is added to AMP-PNP-bound MTs, the modified kinesin still releases the MTs in response to ATP. In contrast in the co-sedimentation assay, modified kinesin shows enhanced binding to MTs in the presence of ATP in a dose-dependent manner. Also in the co-sedimentation assay, equal amounts of modified and unmodified kinesin pellet with MTs in the presence of AMP-PNP. In comparison, modified kinesin shows a reduction of microtubule-stimulated ATPase activity in the ATPase assay.

Why does modification enhance binding in the sedimentation assay while inhibiting binding in the motility assay? At least four possible explanations exist. The first is that kinesin may not be binding to the glass slide in the motility assay; since NEM may modify kinesin in such a manner that it can no longer retain its conformation. This does not appear to be the correct explanation, since NEM treatment either prior to or after kinesin adsorption of glass gives identical results in which microtubule binding is inhibited. A second explanation is that modification "pushes" kinesin over onto the glass, and there it denatures and cannot interact with MTs. Thirdly, the modification may alter the manner in which kinesin interacts with MTs in the sedimentation assay, by altering

previously-existing head-to-tail binding; consequently, interactions with MTs may be enhanced. The fourth and currently-favored explanation concerns the protein conformation in each particular assay. Kinesin can exist in a folded conformation and in an extended conformation (Hackney et al., 1992). All modification is carried out in solution, while the motor is folded. It is likely that in the sedimentation assay kinesin stays in its folded configuration. While kinesin is folded, critical cysteines may be buried in a KHC/KLC interaction. It is also probable that in the motility assay, kinesin is in its extended configuration. While in the extended conformation, the critical cysteines may be available to interact with MTs.

Until this point, most cysteine analysis studies have been done with myosin, dynein, and kinesin proteins, excluding the kinesin-like proteins. One recent study analyzed the reactivity and subsequent conformation of the cysteine residues in the Ncd motor domain (amino acids 335-700). This study, performed by Naber, et al, [1997 #1410] treated the Ncd monomeric motor domain with an NEM-labeled spin probe. Spin probes are often used to identify conformational changes, in this case changes due to interactions with MTs. The majority (80%) of the spin probe labeled only Cys-670. Interestingly, labeling this cysteine did not cause any effects on Ncd motor activity when analyzed in a co-sedimentation assay with MTs. This cysteine is located in an alpha helix that is structurally homologous to the alpha helix of the myosin reactive sulfhydryls. This could imply the location of a reactive-cysteine, since the crystal structures are quite similar. In fact, the cysteine residues line up when three-dimensional structures of myosin and Ncd motor regions are superimposed upon one another (Naber et al., 1997). Judging from Naber's work alone, this cysteine (Cys-670) may be reactive and most readily available in the monomeric structure, but it is still questionable if Cys-670 is critically involved in MT-motor protein interactions.

Chemical modification of Tubulin

The sulfhydryl groups in tubulin have been studied for a role as targets for antimitotic and antimicrotubule chemicals and also for a role in creating asymmetric MTs. Historically, the sulfhydryl groups in tubulin have been used as probes for mediating interactions of tubulin with drugs or other ligands, and are targets for many commonly-used antimitotic chemicals, including: colchicine, nocodazole, vinblastine, and maytansine (Luduena and Roach, 1991). When the sulfhydryls of tubulin were first characterized, tubulin was treated with a variety of sulfhydryl-reactive agents. When treated with [¹⁴C]iodoacetamide, incorporation of ¹⁴C was observed in both α and β tubulin to about the same extent in a time-dependent fashion (Luduena and Roach, 1981a). Tubulin reactivity with iodoacetamide has become the standard to which the other reagents are compared. Treating tubulin with a similar compound, N,N'-Ethylenebis-(iodoacetamide) (EBI), led to the identification of specific cross-linked cysteine residues. This cross-link product was used to

gain a preliminary understanding of the varying reactivities of tubulin's cysteine residues. An intra-chain cross-link forms in β -tubulin between Cys-239 and Cys-354 (Little and Luduena, 1985). The cross-link is easily observed with SDS-PAGE. After the cross-link is formed, the peptide cannot unfold properly and therefore migrates as if it had a smaller molecular weight on SDS-PAGE. When MTs are treated with EBI, less cross-link product is produced, suggesting that these sulfhydryls are not located on the exterior of the MT, and may be buried (Luduena et al., 1982). The cysteines at positions 127 and 129 in β tubulin are highly conserved among mammalian tubulin, and they are no longer exposed after MT assembly. The region containing these residues was postulated to be involved in GTP binding in β tubulin, since it is an exposed loop (de Pereda and Andreu, 1996; de Pereda et al., 1996).

This cross-link product can also be used to analyze the reactions of antimetabolic drugs with tubulin sulfhydryls. Generally, these drugs can inhibit formation of the cross-link product (termed β^*) or they may inhibit the reaction of tubulin with [^{14}C]iodoacetamide. For example, addition of colchicine suppresses formation of the cross-link product. After tubulin has been treated with colchicine, alkylation by [^{14}C]iodoacetamide is inhibited by 33% and alkylation by [^{14}C]NEM is inhibited by 55%, indicating reactions with the same sulfhydryls (Luduena and Roach, 1991). Furthermore, treating MTs with nocodazole, another inhibitor of MT assembly, inhibits binding of colchicine (Hoebeke et al., 1976). Like colchicine, addition of nocodazole also inhibits alkylation of tubulin by [^{14}C]iodoacetamide and blocks formation of the cross-link product, β^* . Interestingly, two other chemical inhibitors of MT assembly, vinblastine and mytansine, both enhance formation of the cross-link product, β^* . Vinblastine strongly inhibits alkylation by [^{14}C]iodoacetamide, while mytansine only weakly inhibits the reaction. Vinblastine also inhibits [^{14}C]NEM alkylation of tubulin by 64% (Luduena and Roach, 1991).

Tubulin sulfhydryls may also play a role in the protein's GTPase activities. Both α and β tubulin bind GTP, and β tubulin hydrolyzes GTP as well. It was postulated that the two most reactive sulfhydryl groups are located close to the binding site(s) of GTP. This is based on the experiments in which tubulin was covalently labeled with a GTP analog, and then treated with NEM (Mann et al., 1978). The binding site of β tubulin is termed the E site, or exchangeable site, where GTP is readily bound and hydrolyzed to GDP + Pi. UV-induced photoaffinity studies showed that GTP cross-links to Cys-12 of β tubulin (Shivanna et al., 1993). The hydrolysis of GTP at the E site is important for the dynamic behavior of MTs, and Cys-12 may play a role in regulating this

behavior. The GTP binding site of α tubulin is termed the N site or the non-exchangeable site, which is deeply integrated into the tubulin structure. Photoaffinity studies identified Cys-295 of α tubulin in close proximity to the N site (Bai et al., 1989).

These experimental findings are summarized in the following statements. The cysteines that react most strongly with GTP, vinblastine, and maytansine are located at the N-terminal half of β tubulin, presumably Cys-12 and Cys-201 or Cys-211. These cysteines are not directly involved with the cross-link product β^* , but have been shown to form a second crosslink product with tubulin that is not as well documented, termed β^s (Luduena and Roach, 1991). Colchicine and nocodazole affect the C-terminal region, Cys-239 and Cys-354, and it is these more reactive cysteines that are involved in formation of the cross-link product, β^* .

Like kinesin, many cysteines in tubulin are also conserved across species. The cross-link products mentioned above are formed in both porcine and bovine tubulin, which are most often used experimentally, and also in tubulin from chicks, fish, squid, brine shrimp, and sea urchin flagella (Detrich et al., 1987; Luduena et al., 1985a; Luduena et al., 1985b; Roach and Luduena, 1984). In fact, Cys-354 is universally conserved among all β tubulin types, indicating that it must play an important role (Little and Seehaus, 1988).

As previously mentioned, MTs exist in a dynamic equilibrium *in vivo* and *in vitro*. Many cellular processes are thought to contribute to this dynamic state, including the state of tubulin's sulfhydryl groups (Mellon and Rebhun, 1976b). It has been shown that sulfhydryl reactive agents disrupt MT assembly in living cells (Nath and Rebhun, 1976; Ramel and Magnusson, 1969). Many experiments point to a single key cysteine that may act to inhibit assembly when oxidized or alkylated (Ikeda and Steiner, 1978; Kuriyama, 1976; Mellon and Rebhun, 1976b). In 1978, Mann et al. [1978 # 1418] observed that assembly was inhibited by NEM-alkylation of one sulfhydryl group per 60 kD tubulin protein. In most reports, several cysteines are labeled simultaneously, and varying cysteines are labeled with different agents; therefore, it has been difficult to localize a single cysteine required for polymerization (Ikeda and Steiner, 1978; Kuriyama, 1976; Mellon and Rebhun, 1976b).

Interestingly, alkylation of the single Cys-239 of β tubulin resulted in complete loss of polymerization (Bai et al., 1989)). This is the only data suggesting that there is one cysteine critical for assembly.

The inhibition of MT assembly due to NEM treatment has been used as a tool in the MT-motor field to determine the direction that a motor protein moves. NEM-treated tubulin alone fails to assemble into MTs (Huitorel, 1988; Hyman et al., 1991). When NEM-treated tubulin is mixed with

untreated-tubulin at appropriate ratios, the normal assembly differences between the plus and minus ends are enhanced. In these MTs, assembly is completely inhibited at the minus end (Hyman et al., 1991). In an early study, NEM-treated tubulin mixed with untreated tubulin in equimolar amounts was shown to inhibit MT assembly by 50% at both ends, compared to assembly of untreated tubulin alone (Deinum et al., 1981). Another study showed that when 5 μ M NEM-tubulin and 14 μ M untreated tubulin were combined assembly at the minus ends was inhibited by >90%, and assembly at the plus end was inhibited by <10% (Mitchison, 1988).

Project Introduction

In order to learn more about MT-motor protein interactions and the mechanisms responsible for tubulin assembly, chemical modification has been employed. As previously described, chemical modification is a widely-used method for analysis of protein-protein interactions. All proteins in this study were treated with N-ethylmaleimide (NEM) which reacts readily with exposed sulfhydryl groups by addition of the mercaptide ion across the protein double bond. NEM is considered to be more specific for sulfhydryls than is iodoacetamide, especially at pH~7 (Luduena and Roach, 1991). This project analyzes the effects of NEM treatment on 1) MT-motor protein interactions of kinesin and Ncd, and 2) tubulin assembly.

NEM-treatment of kinesin has been shown to inhibit binding to MTs in the motility assay (Walker et al., 1997). In contrast, the same assay resulted in enhanced binding to MTs of the related motor protein, Ncd (Walker et al., 1990). The goal of the first project was to examine the reasons behind the opposite effects of NEM on kinesin and Ncd. The modified proteins were assayed for functional differences compared to untreated proteins using co-sedimentation and ATPase assays. To gain further insight into kinesin and Ncd motor activity several proteins were treated in solution with a range of NEM concentrations (0 - 10 mM). The proteins used in this study are two *Drosophila* kinesin constructs (DK350 and Trx-DK375), one squid construct (p181), and the Ncd motor-stalk construct MC1 (Modified Claret 1, (Chandra et al., 1993). DK350 is a monomeric protein, containing only the N-terminal motor domain. Trx-DK375, MC1, and p181 are all dimers. Results of NEM-treatment varied for these proteins. NEM had no effect on the ability of the kinesin proteins to bind MTs in the co-sedimentation assay. Co-sedimentation experiments with MC1 demonstrated that lower concentrations (< 0.1 mM) of NEM enhanced the binding of MC1 to MTs in the presence of ATP, but that higher concentrations (> 0.5 mM) of NEM induced apparent aggregation of the MC1 protein. Treatment with concentrations up to and including 10 mM NEM reduced both the basal and MT-stimulated ATPase rates of DK350, but not those of Trx-DK375. NEM concentrations as high as 5 mM had no effect on the basal ATPase rate of MC1, but there was a dose-dependent inhibition of MC1's MT-stimulated ATPase activity.

The objective of the second project was to gain a better understanding of how NEM effects minus-end tubulin assembly. Tubulin dynamics were observed using video-DIC microscopy. Varying concentrations of NEM were used to treat tubulin to determine the concentrations necessary for inhibition of minus-end assembly. In a similar manner, untreated tubulin plus NEM-treated tubulin ratios of 1:1, 2:1, and 4:1 were assayed for effects on minus-end assembly. Replacement experiments, performed in flow cells where solutions are easily changed, were used to observe differences between the effects of NEM-treated tubulin on plus and minus end assembly. Finally, in both projects, protein digestion techniques were used to better determine the location of reactive cysteines.

Materials & Methods

Protein Preparations

All motor proteins were purified in a similar manner. The pET3/MC1 (Chandra et al., 1993), pET21/DK350, pET32/DK375, and pET29b/p181 (Kosik et al., 1990) constructs were transformed into BL21(DE3) or BL21(DE3)pLys *E.coli* cells. Protein expression was induced by addition of 0.25 mM IPTG. After four hours at 24-25°C, cells were pelleted and washed in AB Buffer (20 mM Pipes, pH 6.9, 1 mM MgSO₄, 1 mM EGTA) with or without 5% sucrose (AB/sucrose). Cell pellets were frozen and stored at -70°C. Frozen cells were resuspended in 3 ml per gram of AB/sucrose or phosphate buffer (10 mM phosphate pH 7.2, 0.1 M NaCl, 2 mM MgCl₂, 1 mM EGTA) plus 5% sucrose (PB/sucrose). BL21(DE3) cells were lysed by addition of 0.2 mg/ml lysozyme and one freeze/thaw cycle, then DNase I and MgCl₂ were added to 40 mg/ml and 10 mM respectively. Only DNase I and MgCl₂ were added to BL21(DE3)pLys cells. After 30 minutes on ice, lysates were centrifuged at 20,000xg for 15 minutes (4°C). Low speed supernatants were subsequently centrifuged at 100,000xg for 15 minutes (4°C), and the high-speed supernatant was fractionated by S-Sepharose ion exchange chromatography. Proteins containing a His-Tag sequence (DK375 and p181) were fractionated as described, and further purified with Talon (Clontech) metal affinity resin. Bound protein was eluted with AB, AB/sucrose, or PB/sucrose containing 0.2 M or 0.25 M NaCl. MgATP (0.1 mM) was present throughout the purification process, while PMSF (1 mM) and DTT (1 mM) were added to the lysis and column wash buffers but omitted from the elution buffer. Eluted proteins were dialyzed at 4°C against AB or AB/sucrose plus 0.1 mM MgATP, then quick frozen in liquid nitrogen and stored at -70°C.

Tubulin was purified from porcine brain by two cycles of polymerization and depolymerization in PM buffer (100 mM Pipes, 2 mM EGTA, 1 mM MgSO₄, 1 mM GTP, pH 6.9), followed by passage over a phosphocellulose column (Williams and Lee, 1982). After collection from the column, the tubulin was subjected to an additional round of sodium-glutamate-promoted assembly, then quick frozen in liquid nitrogen and stored at -70°C (Walker et al., 1988). Tubulin in PM buffer was depleted of DTT by passage over a PD-10 column (Pharmacia). These samples were also quick frozen in liquid nitrogen and stored at -70°C. The purity of the tubulin prep was examined by Coomassie-blue staining of protein separated on SDS-PAGE gels.

All protein concentrations were determined with a Bradford colorimetric assay (BioRad) using BSA as a standard. MC1, DK375, and p181 concentrations are expressed as monomer concentrations.

Protein Modification and Functional Assays

Stock solutions of NEM (Sigma) or Biotin-NEM (Molecular Probes) were prepared fresh as 5X stock solutions in AB or AB/sucrose. Motor proteins (7.5 μ M final) were modified with 0.05, 0.1, 0.5, 1, 5, or 10 mM NEM, yielding molar ratios of 6.5:1, 13:1, 66:1, 133:1, 666:1, and 1333:1 respectively. Control modification reactions were prepared by the addition of buffer alone. Motor proteins were modified at room temperature (21°C) for up to 30 minutes. Reactions were quenched by the addition of DTT (50 mM final) then placed on ice for 10-15 minutes prior to use.

To examine MT binding in a co-sedimentation assay, modified and control motor samples (2.5 μ M final) were mixed with taxol-stabilized MTs (2.5 μ M tubulin final) in AB or AB/sucrose and either MgATP or MgAMP-PNP (5 mM final). Identical reactions were prepared without MTs to evaluate non-specific protein pelleting. After 30 minutes at room temperature, samples were centrifuged at 100,000xg, and supernatant and pellet fractions were then analyzed by SDS-PAGE (7.5% gels).

MT-stimulated activities of DK350 and MC1 were determined using the pyruvate kinase ATPase assay. Proteins were modified as described above. The ATPase reaction was initiated by addition of motor protein (50 nM final) to a cuvet containing 50 mM Tris-acetate buffer, plus 2 μ M MTs, 20 μ M taxol, 1 mM MgATP, 1 mM DTT, 3 mM phosphoenol pyruvate, 7.5 U pyruvate kinase, 12.5 U lactate dehydrogenase, and 0.2 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm over time using a Beckman DU640 spectrophotometer. Basal and MT-stimulated activities of DK350, DK375, and MC1 were also determined using a malachite green phosphate assay (Baykov et al., 1988). All reactions contained 0.1 μ M motor protein and MT-stimulated reactions contained GTP-depleted taxol-stabilized MTs (2 μ M final). Reactions were initiated with the addition of 1 mM MgATP and allowed to proceed for a total of 25-30 minutes with samples taken at 5 minute intervals. MT-stimulated reactions were first quenched by addition of an equal volume of 0.5 M HCl and were then centrifuged to pellet precipitated protein. Reaction samples were mixed with malachite green-molybdate color reagent at a 4:1 ratio (v/v) of protein sample to malachite dye reagent (Baykov et al., 1988). After a 10 minute incubation at room temperature, the absorbance was measured spectrophotometrically at 630 nm.

Identification and Characterization of Reactive Cysteines in Motor Proteins

DK375, p181, and MC1 (7.5 μ M) were treated with 0.05, 0.1, 0.5, 1, and 5 mM [3 H]NEM (either 2.6×10^{13} or 3.4×10^{13} cpm/mol). For each concentration, samples were taken at 5, 10, 20, and 30 minutes and the modification reactions were quenched by addition of DTT (100 mM final). Samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Protein bands

were excised from the gel and solubilized with 1 ml 30% H₂O₂ for 24 hours at 60°C. Each dissolved gel slice was then mixed with 9 ml scintillation fluid and counts per minute (cpm) were measured.

For further characterization of reactive cysteines, MC1 protein was subjected to various methods of digestion to identify the reactive cysteine(s). MC1 was modified with biotin-NEM as described above and modified protein samples (40 µg) were precipitated by a chloroform-methanol procedure (Wessel and Flugge, 1984). The dried protein pellets were resuspended in 20 ml of 88% formic acid (Fisher) and incubated for 24 hours at 37°C. Samples were then diluted by addition of 480 µl distilled water and Microcon-3 or -10 devices (Amicon) were used to concentrate the protein. Two volumes of 1 M Tris was added to the concentrated protein sample, and 6X sample buffer (0.28 M TrisHCl pH 6.8, 30% glycerol, 1% SDS, 0.5 M DTT, 0.0012% bromophenol blue) was added to the protein samples. Formic acid digested MC1 samples were separated by SDS-PAGE (12.5% gels) or Tricine gel electrophoresis (10% gels), followed by Western blot analysis. Biotin-NEM labeled fragments were detected by an anti-biotin primary antibody (Sigma) (1:2,500) and an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:10,000), while MC1 bands were detected with a polyclonal antibody (VP76) (1:2000) raised in rabbit against the motor domain (MC6) of Ncd followed by an alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:10,000). Reactive bands were detected with the Vistra ECF blotting reagent (Amersham) on a Storm imager (Molecular Dynamics).

For identification of reactive cysteine residues in tubulin, digestion with endoproteinases Lys-C and Trypsin (Boehinger-Mannheim) was used. Endoproteinases were added at a 1:50 or 1:25 enzyme to protein weight-to-weight ratio. Protein digestion proceeded for 24 hours at 37°C and was stopped by freezing (Lys-C) or heating to 85°C (Trypsin). Protein fragments were analyzed using an HPLC separation system (Applied Biosystems) over a 50% acetonitrile concentration gradient.

Axoneme Preparation

Flagellar axoneme fragments were prepared from sea urchin, *Lytechinus pictus*, according to the method of Bell et al.[1982 #376]. Axonemes were osmotically demembrated and mechanically separated from sperm heads by homogenization in a solution of 20% sucrose in distilled water, using a handheld Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Axonemes were resuspended and washed in isolation buffer composed of 0.1 mM NaCl, 4 mM MgSO₄, 1 mM EDTA, 7 µM β-mercaptoethanol, and 10 mM Hepes (pH 7.0). Dynein outer arms were removed by incubation in isolation buffer and adjusted to 0.6 M NaCl for 30 min at 4°C. Residual sperm heads were found to enhance both nucleation and elongation and were therefore removed by sedimentation of the axoneme

preparation through an 80% sucrose cushion (16,000g, 10 min.). Axonemes were stored at -20°C in a 1:1 solution of isolation buffer/glycerol. Axonemes were washed and resuspended in PM before use.

Modification of Tubulin

NEM modification of tubulin proceeded for up to 30 minutes on ice (0°C). NEM stock solutions (5x) were prepared fresh in PM buffer. Control samples were prepared by addition of PM alone. Tubulin (25 μM final) was modified with 0.025, 0.05, 0.1, 1, or 5 mM NEM, yielding molar ratios of 1:1, 2:1, 4:1, 40:1, and 200:1 respectively. Reactions were quenched by addition of 50 mM DTT, and placed on ice before use in assembly reactions. NEM-treated tubulin is termed NTb, while untreated, phosphocellulose-purified tubulin is termed PCTb.

Characterization of Reactive Cysteines in Tubulin

Tubulin or taxol-stabilized-microtubules (25 μM) were treated with 0.025, 0.05, 0.1, 0.5, 1, and 5 mM [^3H]NEM (either 2.6×10^{13} or 3.4×10^{13} cpm/mol). For each concentration, samples were taken at 5, 10, 15, and 30 minutes and the modification reactions were quenched by addition of DTT (100 mM final). Samples were separated by SDS-PAGE using low-grade SDS (95% pure) and stained with Coomassie Brilliant Blue. Impurities in the low-grade SDS allow α and β tubulin to separate on the gel. α and β tubulin bands were excised from the gel and solubilized with 1 ml 30% H_2O_2 for 24 hours at 60°C . Each dissolved gel slice was then mixed with 9 ml scintillation fluid and counts per minute (cpm) were measured.

Assay of Microtubule Dynamics

Microtubule dynamics were analyzed in slide chambers, using axonemes as sites for nucleation. The axoneme and GTP (1 mM) concentrations were held constant for all preparations. A 10 μl sample of the prepared axonemes was added to a clean slide covered with a biologically clean 22- mm^2 coverslip (thickness No. 0) placed on double-sided tape. The typical separation between the inner glass surface was 10-20 μm . The slides were observed in a room held at $23\text{-}24^{\circ}\text{C}$. Once added to the slide-coverslip chamber, axonemes were incubated for three minutes and then those that did not adhere to the glass were washed out of the chamber with PM buffer. Axonemes tightly adhere to the clean glass surfaces of the chamber, while MTs and free tubulin subunits do not adhere to the glass as proven by various methods (Wadsworth and Salmon, 1986; Walker et al., 1988).

Microtubules assembled onto sea urchin axoneme fragments were identified as plus or minus ended based on rate of elongation. Tubulin was assembled off axonemes or assembled off MTs in condition/solution-replacement experiments. Untreated tubulin was mixed with NTb in solution in ratios of 1:1, 2:1, or 4:1, before being added to the chamber. Tubulin samples were kept on ice and then mixed immediately prior to use.

Video Microscopy and Assembly Analysis

Microscopic methods were based upon those reported by Pryer et al [1986 #422]. Preparations were viewed by DIC microscopy, using a Nikon SA photomicroscope equipped with a Plan 60x/1.4 NA oil immersion objective lens, DIC prisms, and a 1.4 NA condenser. A 100 W mercury lamp and fiber optic scrambler provided full illumination of the condenser aperture through heat-cut and 546-nm interference filters. Image magnification at the video camera faceplate was 350x. Image contrast was enhanced by analog and digital video processing (Allen et al., 1981; Inoue, 1981). Video image generation and analog contrast enhancement were provided by a Dage VE-1000 newvicon video camera. An Argus 10 processor provided further contrast enhancement, real-time background subtraction, and frame averaging. Images were recorded on a Panasonic SuperVHS videocassette recorder.

Data Analysis

Microtubule elongation and rapid shortening rates were measured from videotape recordings. A computer-based analysis system was used to follow microtubule length changes in real time (Walker et al., 1988). A point cursor was electronically overlaid on the video screen and used to track the end of the microtubule. Changes in microtubule length were plotted as a function of time, and the average rates of elongation and rapid shortening were determined by least square regression analysis.

Results I

Results of NEM Treatment on MT-Motor Proteins

Each protein was studied individually and analyzed for effects due to NEM-treatment. Full length *Drosophila* kinesin contains 8 cysteines (Yang et al., 1988). The kinesin constructs DK350, a monomer, and the fusion protein Trx-DK375 (from this point referred to as DK375), each contain 5 cysteines. Thioredoxin adds two additional cysteines to DK375, which is believed to exist as a dimer (Jiang et al., 1997). DK375 contains the full motor domain and part of the stalk domain, up through amino acid 375. The squid construct, p181, is a 67.8 kD protein of 602 amino acids, and contains 5 of the 8 cysteines present in full-length squid kinesin. MC1 contains residues 209-700 of the minus-end directed, C-terminal-motor Ncd protein and includes nine of the eleven cysteines present in full length Ncd. MC1 and p181 both contain the full motor and stalk domains of the parent proteins.

Effect of NEM on Motor Protein Binding to MTs and ATPase Activities

NEM was previously shown to inhibit binding of native chick kinesin to MTs (Walker et al., 1997). To characterize the effect of NEM on the MT-binding properties of each protein, DK350, DK375, p181, and MC1, (7.5 μ M) were treated with varying concentrations (0-10 mM) of NEM for 30 minutes. Modification reactions were stopped by addition of DTT (50 mM final) to quench unreacted NEM, and subjected to MT co-sedimentation in the presence of either 5 mM MgATP or 5 mM MgAMP-PNP. In the co-sedimentation assay, unmodified motor proteins bind to MTs in the presence of MgAMP-PNP, and are found in the pellet fraction. While in the presence of MgATP, unmodified motor proteins release MTs and are found in the supernatant fraction. Without any nucleotide present, unmodified motor proteins bind to MTs and are held in rigor, identical to the results caused by AMP-PNP; therefore, this condition was not analyzed. Supernatant and pellet fractions from SDS-PAGE were stained with Coomassie blue for both DK350 and DK375 in order to analyze the effects of NEM-modification on MT-binding. NEM-treated DK350 did not pellet in the absence of MTs. In the presence of MgAMP-PNP, a non-hydrolyzable analogue of MgATP, DK350 bound to the MTs and this binding was not significantly affected by 1 or 10 mM NEM. In the presence of MgATP, DK350 released from the MTs and was found in the supernatant fraction. DK375 was also analyzed since it has a greater stalk region, and is believed to exist as a dimer (Jiang et al., 1997). Modified DK375 acted like DK350 in that both bound to MTs with added MgAMP-PNP and released MTs with added MgATP. DK375 was also treated with NEM at 37°C, and there was no inhibition of MT-binding in the co-sedimentation assay at this modification temperature. Thus, neither *Drosophila* kinesin protein was affected by any concentration of NEM used.

The MT binding of squid kinesin, p181, was also assayed in the co-sedimentation assay, and results analyzed by Coomassie blue stained SDS-PAGE. In the absence of MTs, 14% \pm 3% p181

was found in the pellet fraction. In the presence of MgATP, $37\% \pm 3\%$ p181 pelleted, while the majority of the protein released from MTs and was found in the supernatant fraction. With MgAMP-PNP added, the majority of p181 bound MTs; $70\% \pm 9\%$ p181 was found in the pellet fraction. These numbers did not change dramatically with NEM-modification. The percent of modified p181 in the pellet was as follows: in the absence of MTs $30\% \pm 5\%$; plus MgATP $42\% \pm 4\%$; plus MgAMP-PNP $73\% \pm 8\%$. Modification of p181 at 37°C gave similar results to those performed at 25°C . Thus, unlike native kinesin (Pfister et al., 1989; Walker et al., 1997), NEM modification did not enhance the binding of DK350, DK375, or p181 in the co-sedimentation assay.

The kinesin-like protein, MC1, was analyzed with the co-sedimentation assay and Coomassie blue stained SDS-PAGE. Gels were quantified using the gel documentation system to determine the proportion of MC1 present in the pellet fractions. Control MC1 samples that were not treated with NEM but that received DTT acted as expected based on previously reported co-sedimentation experiments (Chandra et al., 1993). Relatively little unmodified MC1 was present in the pellet fraction in the absence of MTs (24%) or in the presence of MTs and MgATP (27%), while the majority of unmodified MC1 (68.5%) was found in the pellet fraction in the presence of MTs and MgAMP-PNP. Treatment of MC1 with 0.05 or 0.1 mM NEM only slightly increased the amount of MC1 that pelleted in the absence of MTs (to 29.5% and 36% respectively) and in the presence of MTs and MgAMP-PNP (to 78% and 85.5% respectively). However, in the presence of MTs and MgATP, the amount of 0.05 or 0.1 mM NEM-modified MC1 in the pellet fraction was significantly increased relative to the unmodified control sample (from 27% to 77 and 78% respectively). This indicates enhanced binding of the modified MC1 to MTs under conditions that would normally release the motor to the supernatant fraction. When the NEM concentration in the modification reaction was increased to 0.5 mM, the modified MC1 was found almost completely (70% or greater) in the pellet fraction regardless of whether MTs were absent or present. Similar results were also observed when MC1 was modified with 1, 5, or 10 mM NEM. Thus, when MT binding was assayed by co-sedimentation, treatment of MC1 with NEM produced two different effects depending on the NEM concentration used. Lower NEM concentrations enhanced binding of the modified motor to MTs in the presence of MgATP, while higher doses led to aggregation of the modified motor and consequently the inability to evaluate the effect of higher NEM doses upon the MT binding activity of MC1.

To examine further the NEM-induced motor aggregation, MC1 was treated with NEM (0.05, 0.1, 0.5, 1, 5, or 10 mM) and samples were collected over a 30 minute period and centrifuged in the absence of MTs. MC1 pelleting in the absence of MTs occurred in both an NEM dose-dependent manner and a time-dependent manner. When MC1 was treated with either 0.05 or 0.1 mM NEM, there was a slight increase in the proportion of MC1 that pelleted over the 30 minute time course

(from <20% at 5 minutes to 35% at 30 minutes). At NEM concentrations of 0.5 mM, the majority of MC1 (55%) was present in the pellet fraction by 10 minutes and this proportion changed little over the remaining 20 minutes of the reaction. NEM concentrations of particular interest were analyzed for enhanced binding activity or protein aggregation over a time course. MC1 treated with 0.1 mM NEM shows enhanced binding to MTs after only 5 minutes. This is indicated by the amount of MC1 in the pellet in the presence of MgATP, which is greater than that found in the pellet in the presence of MgAMP-PNP compared to the control.

Next the effect of NEM on the ability of the motor protein to hydrolyze ATP was examined. Modifications were performed as described in Materials and Methods, and the effects of NEM treatment on the MT-stimulated ATPase rates were determined. The effect of NEM on the MT-stimulated ATPase rates of DK350 and MC1 was determined using the Pyruvate Kinase ATPase assay. This assay was performed as explained in Materials and Methods. The results showed a decrease in MT-stimulated ATPase rates of modified motors, compared to control motors. However, the DTT that was added to quench the NEM reaction interfered with the coupled enzymes of the assay leading to an apparent decrease in the ATPase rate, independent of NEM treatment. Rates reported are relative to control rates of motors treated at room temperature with added DTT. Treatment with 1 mM NEM reduced the MT-stimulated rate of DK350 by 33% and 10 mM NEM reduced it by 67%. Treatment with 0.05 mM NEM reduced the MT-stimulated ATPase rate of MC1 by 25%, 0.1 mM NEM reduced it by 44%, and 0.5 mM NEM reduced it by 73%.

Due to the interference of DTT described above, MT-stimulated and basal rates were determined with a second assay, the Malachite Green ATPase assay. Control samples of proteins that were not treated with NEM but that received DTT behaved as expected both in terms of basal and MT-stimulated rates. The basal rate of DK350 was decreased by 50%, from 0.12 ± 0.03 ADP/s (n=11) to 0.06 ± 0.02 ADP/s (n=7). However, the basal rate of DK375, 0.09 ± 0.03 (n=5) was not significantly affected by 10 mM NEM treatment, 0.07 ± 0.03 (n=5). The MT-stimulated rate of DK375 was determined to be 36.0 ± 11.2 ADP/s (n=9), an increase of 400-fold compared to the basal rate. After treatment with 10 mM NEM, this rate was determined to be 34.0 ± 11.0 (n=4) ADP/s. There was no significant difference between the modified and control MT-stimulated ATPase rates of DK375. The basal rate of unmodified MC1 at 22°C was 0.05 ± 0.02 ADP/s (n=15), and that rate was increased about 28-fold to 1.40 ± 0.21 ADP/s (n=13) upon addition of MTs. Treatment of MC1 with NEM concentrations as high as 5 mM had no effect on the motor's basal rate, but did produce a dose-dependent decrease in the MT-stimulated ATPase rate of MC1. The MT-stimulated rate of MC1 after treatment with 0.1, 0.5, 1 and 5 mM NEM was decreased by 21%, 46%, 57%, and 70% respectively. Thus enhancing binding did not result in an increase in the ATPase rate of the motor protein, and some MT-stimulated ATPase activity persisted even under conditions where the majority of the protein pelleted.

Characterization of Reactive Cysteines in Kinesin

Although DK375 and p181 did not show effects of NEM treatment in the co-sedimentation or ATPase assays, each protein was treated with [³H]NEM in order to characterize any reactive cysteines. Not surprisingly the numbers for reactive cysteines are low for both DK375 and p181. Both proteins were treated with 1 mM and 5 mM [³H]NEM over a time course of 30 minutes. At the end of the reaction period, an average of 0.82 ± 0.14 cysteines were labeled in DK375 with 1 mM NEM. This number only slightly increased with 5 mM NEM, to an average of 1.24 ± 0.11 cysteines. Since these are population assays, the majority of proteins may have one cysteine labeled while others do not have any labeled, and perhaps a few have two labeled. It is possible that the labeled cysteine is from thioredoxin (Trx) in the Trx-DK375 fusion protein. Similarly with p181, after 30 minutes an average of 0.27 ± 0.04 cysteines with 1 mM, and an average of 0.32 ± 0.05 cysteines with 5 mM were labeled. These numbers suggest that the majority of p181 protein was not labeled at all. Perhaps cysteines involved in effects observed with native kinesin (Walker et al., 1997) are found in the tail or in light chains, or were not easily modified in these assays, which were done at 24°C. Since there was no obvious functional effect of modification on these proteins, no further characterization of reactive cysteines was performed.

Characterization of Reactive Cysteines in MC1

To correlate the observed effects on the MT binding, solubility, and ATPase activity of MC1 with modification of specific cysteines, the number of NEM-modified residues per protein was determined over a time course of 30 minutes. MC1 (7.5 μM) was treated with 0.05, 0.1, 0.5, 1.0, or 5.0 mM [³H]NEM, and samples were taken at 5, 10, 20, and 30 minutes at each concentration. After treatment with 0.05 or 0.1 mM NEM for 10 minutes, an average of 1 cysteine residue was modified per MC1 protein, and this ratio increased to an average of 1.4 and 1.6 cysteines per MC1 after 30 minutes for 0.05 mM and 0.1 mM respectively. In comparison, treatment of MC1 with 0.5 mM or 1 mM labeled an average of approximately 2 cysteines per MC1 protein by 10 minutes and labeling appeared to reach a plateau of an average of 2 cysteines per MC1 by 20 minutes. At the highest NEM concentration used in these experiments (5 mM), an average of 2.1 cysteines were labeled after 5 minutes and this ratio increased only slightly (to 2.3 cysteines/MC1) after 30 minutes. Thus, at the maximum ratio of >600:1 used in these modification reactions, the maximum number of cysteines that could be labeled was an average of approximately 2 (of the 9). These provided data

suggest that one critical cysteine may be involved in the enhanced MT binding and modification of a second cysteine may be involved in the aggregation of MC1.

Modified MC1 in a Motility Assay

Unlike other motor proteins, MC1 is not capable of normal directional movement; however, it does exhibit one-dimensional diffusional movement (Chandra et al., 1993). This diffusional movement was observed in the presence of ATP and AMP-PNP, using low concentrations of MC1 (1 μ M). Modification with 0.1, 1, 5, or 10 mM NEM had no apparent effect on this diffusional movement.

Identification of Reactive Cysteines in MC1

MC1 was next labeled with biotin-NEM to facilitate identification of the specific modified cysteines. Modification of MC1 with biotin-NEM was found to produce results identical to those observed for NEM both in terms of enhanced MT binding at lower biotin-NEM concentrations and induced aggregation at higher biotin-NEM concentrations. Biotin-NEM-modified MC1 was initially subjected to digestion with formic acid, which preferentially cleaves peptide bonds between aspartic acid and proline. MC1 contains one such formic acid-sensitive site between amino acids Asp-524 and Pro-525, in Ncd (these are MC1 AA 315/316), and formic acid digestion should yield one 36 kD fragment and one 19 kD fragment. MC1 was treated with 0.1 or 1.0 mM biotin-NEM for 10 minutes. Based on the data obtained with [3 H]NEM, these modification conditions would be expected to label one and two cysteines respectively. After formic acid digestion, samples were separated by SDS-PAGE, and stained by Coomassie blue dye or transferred to nitrocellulose, and probed with antibodies against biotin and MC1. Coomassie blue staining showed 2 major products of the expected size in control and biotin-NEM-modified formic acid digests. Modification was evident in a qualitative sense based on the shift of the uncleaved biotin-NEM-treated protein to a higher apparent molecular weight. The anti-biotin antibody only recognized the larger fragment (and undigested MC1). No biotin labeling was detected on the smaller fragment or on any digestion intermediates. To evaluate the transfer of both fragments, samples were also treated with antibody VP76 (raised against the Ncd motor domain), that recognized both major fragments and undigested MC1 protein (data not shown). The results of this experiment suggest that the critical cysteine(s) are present between Ncd residues 209 to 524 (excluding the two most C-terminal cysteine residues).

MC1 samples modified with biotin-NEM were then digested with endoproteinase Lys-C and trypsin to generate peptides for sequence analysis by mass spectrometry, in order to better characterize reactive cysteines. Unfortunately, the potential NEM-reactive cysteines were not further characterized at this point. The identification of NEM-reactive cysteines is still in progress.

Discussion I

Effects of NEM on Kinesin Proteins

NEM treatment was previously found to inhibit the binding of native kinesin to MTs in the co-sedimentation assay (Walker et al., 1997). However, unlike native kinesin, there was no observed effect with the kinesin constructs used in this study in the same assay. The monomeric kinesin, DK350, showed a decrease in basal and MT-stimulated ATPase rates, but the dimeric protein, DK375, did not show these effects. Perhaps NEM treatment does not affect the rates of DK375 since it is a dimer; i.e., the stalk region may play a role in stabilizing ATP hydrolysis, regardless of NEM modification. Alternatively, in the three-dimensional protein conformation, the stalk may protect reactive cysteines and prevent modification of critical cysteines by NEM. This idea is supported by the fact that NEM treatment did not affect the basal or MT-stimulated ATPase rate of the dimer DK375. Observed effects on native kinesin were obtained from assays performed at 37°C. It is believed that the increased temperature helps to open the protein, letting it “breathe” and thereby making reactive cysteines more accessible (Walker et al., 1997). However, no effects were observed for DK350 or DK375 with modification performed at 37°C. The reactive cysteines required to observe an effect, like that observed with native kinesin, simply may not be present in DK350, DK375, or p181. It is also possible that effects observed with native kinesin were due to modification of the kinesin heavy chain domain and/or kinesin light chains, neither of which are present in DK350, DK375, or p181. When the protein is folded in solution, as in the co-sedimentation and ATPase assays, cysteines in the tail or light chains may be important. In conditions such as those used in the co-sedimentation and ATPase assays, kinesin is thought to fold so that the tail is brought close to the head. Thus modification of the tail domain and/or light chains may have an effect on motor domain function. Additionally, the tail domain may be important due to the fact that it has homology to the region in myosin containing the two reactive sulfhydryl groups, observed in the squid and *Drosophila* kinesin tail regions (Kosik et al., 1990). This region includes Cys-859 to Ala-870 in squid, and there is a complementary region in *Drosophila* (Kosik et al., 1990). The fact that these regions are conserved is an important detail, implying a degree of importance for particular residues. There are eight conserved cysteines found in *Drosophila* and squid kinesins. The location of one cysteine residue on the C-terminal tail domain between residues

850-860 is conserved among nearly all sequenced kinesin proteins, including squid, sea urchin, mouse, and human. A cysteine in a similar location in *Drosophila* kinesin is located just outside of this region, at position 908. Future analysis of kinesin tail domain constructs could determine if any of the cysteines in the tail are reactive and important.

Effects of NEM on MC1

NEM-treatment of full-length recombinant Ncd (1-700 AA) has previously been shown to result in an enhanced binding to MTs in the motility assay (Walker et al., 1990). MC1 contains the full head and stalk domains (209-700 AA), and exists as a dimer. NEM treatment has two different effects on MC1, depending on the NEM concentration used for modification. As observed with its parent protein Ncd, treatment with < 0.1 mM NEM resulted in an enhanced binding of MC1 to MTs as observed in the co-sedimentation assay. It was not possible to detect any apparent enhanced binding in the motility assay, as was done with Ncd, since MC1 does not exhibit directional movement. Based on [³H]NEM results, this enhanced binding of Ncd occurs with only one cysteine labeled.

At >0.1 mM NEM, MC1 presumably aggregates and this results in protein pelleting in a co-sedimentation assay. This aggregation correlates with the labeling of more than one cysteine per MC1 protein based on [³H]NEM results. MC1 starts to pellet with 0.5 mM NEM treatment. After 10 minutes with this treatment, the protein was distributed equally between supernatant and pellet fractions. Even only 20 minutes after treatment, 77% of the protein was found in the pellet fraction in a co-sedimentation assay. Based on an average of 1.5 cysteines labeled, one possibility is that the majority of MC1 pellets when half of all the MC1 proteins have an average of 2 of the 9 cysteines labeled, while the other half presumably have only an average of one cysteine labeled.

With 1 and 5 mM NEM treatment, which is up to a 500-fold excess NEM over protein, only 2 cysteines were labeled. This could imply that the protein conformation is not severely altered, which might expose other cysteines. This idea correlates with the fact that MC1's basal ATPase rate is not altered by NEM treatment at these concentrations. Labeling of these cysteines does not directly affect the location of ATP binding nor ATP hydrolysis alone. As the second cysteine is labeled, aggregation occurs and interactions with MTs are altered as shown by changes in co-sedimentation and MT-stimulated ATPase rate assays; however, this does not affect the basal ATP hydrolysis rate. When >50% of MC1 has 2 cysteines labeled there is a loss of MT-stimulated ATPase rate by 50%. Perhaps the labeling of the second cysteine blocks an important MT interaction by MC1.

This data can be correlated with the location of cysteines in the three-dimensional conformation of Ncd determined by x-ray/electron crystallography (Kull et al., 1996). Formic acid digestion and immunoblotting ruled out the two most C-terminal cysteines, 670 and 653. Interestingly, it was Cys-670 that was labeled with the spin probe by Naber et al [1997 #1410];

however, this study was done using a monomeric protein and the stalk region may protect or cover the region of the motor domain in the dimeric protein MC1. Using computer modeling programs it is possible to analyze the location of particular amino acids in relation to other areas of interest.

Cys-469 is close to the presumed MT binding loop 12 (AA 620-623). In fact Cys-469 is in direct contact with Arg-623. The phosphate-binding loop contains AA 434-442, and no cysteines are in close contact with this loop. This last point is consistent with the finding that NEM modification does not affect the basal ATPase rate.

The neck region is currently being studied as an important determinant for directionality through a conformational change by amplifying motion towards one end of the MT. In a recent study, the Ncd dimer, including two heads, the neck and part of the stalk, was analyzed using molecular replacement methods/mutagenesis and crystallography experiments (Sablin et al., 1998). This study analyzed the region including amino acids 303-672 from each of two heavy chains (8 cysteines). The short neck region of Ncd is highly conserved among the kinesin superfamily, especially amino acids 335-347. Results of this study included analysis of the neck region that interacts directly with the catalytic motor domain to within 690 angstroms (neck: AA 335-346, core: AA 420-640). Similarly, part of the stalk domain was also shown to interact with the catalytic core (stalk: AA 325-333, core: AA 471-567). The importance of this study in relation to the cysteines found in MC1 is the fact that this neck/stalk region, found to interact with the core, is bordered by two cysteines. Cys-324 ends the stalk region and has not yet been shown to directly interact with the catalytic core. Cys-353 is located just outside the neck on the other end, but is not likely to interact with the catalytic core. The region of the neck that interacts with the motor ends with Arg-346. The direct importance of these cysteines is not understood at this time, but these residues may play some role in regulating the conformation of the MT-binding loop 12 and its relations to the neck.

Results II

Results of NEM Inhibition on Tubulin Assembly

As previously discussed, the role of sulfhydryl groups in tubulin assembly has been an active area of research since the mid-1970s. More recently, NEM modification of tubulin has been used as a tool in the MT-motor field to determine the directionality of motor movement. Although NEM-treated tubulin alone is unable to assemble, when NEM-treated tubulin is mixed with untreated tubulin, plus end MTs assemble at almost normal rates while minus end assembly is significantly inhibited (Huitorel, 1988; Hyman et al., 1991).

The goal of this study was to gain a better understanding of the mechanism by which NEM-treated tubulin enhances the assembly differences between the plus and minus ends. The porcine α tubulin subunit has 12 cysteine residues (451 AA). The porcine β tubulin subunit has 8 cysteine residues (445 AA). Of these 20 total cysteines perhaps only a few may be responsible for the observed effects resulting from NEM-treatment.

Characterization of Reactive Cysteines in Tubulin

To correlate the effects of NEM modification with the extent of cysteine modification, the number of NEM-modified residues per α and β tubulin was first determined over a time course using [3 H]NEM. Tubulin and taxol-stabilized MTs (25 μ M) were treated with 0.025, 0.05, 0.1, 1.0, or 5.0 mM [3 H]NEM, and samples were taken at 5, 10, 15, and 30 minutes at each concentration. After treatment with 1 mM NEM for 10 minutes, an average of 1 cysteine residue was modified on each α and β tubulin, and this ratio increased to an average of 1.5 cysteines per monomer after 30 minutes. Treatment with 5.0 mM NEM for 30 minutes labeled an average of 1.5 cysteines on α tubulin after 15 minutes and an average of 2 cysteines on α tubulin after 30 minutes, while treatment with 5.0 mM NEM labeled an average of 1.7 cysteines on β tubulin after 15 minutes and this number remained at an average of 1.7 cysteines even after 30 minutes.

For comparison, taxol-stabilized MTs (TMTs) were treated in an identical manner to see if any NEM-reactive cysteines were buried after MT assembly. Treatment with NEM concentrations of <5 mM, labeled an average of ≤ 0.3 cysteines on both α and β tubulin after 60 minutes. Treatment with 5.0 mM NEM labeled an average of 1 cysteine on α tubulin by 30 minutes and an average of 2 by 60 minutes, and labeled an average of <1 cysteine on β tubulin by 30 minutes and an average of 1 cysteine by 60 minutes. TMTs were treated for a total of 60 minutes to allow NEM to react fully with

any available sulfhydryls, and still only 2 cysteines on α and one on β were labeled. The reactive cysteines in tubulin, specifically one on β tubulin, may be masked once assembled into MTs.

Effects of NEM on Tubulin Assembly

After determining the number of cysteines labeled, various NEM concentrations were tested for their effects on tubulin assembly onto axonemes adhered to glass coverslips in prepared flow cells. Elongation and dilution-induced shortening rates were determined for MTs assembled from a 2:1 ratio of untreated tubulin (25 μ M) and NEM-treated tubulin (12.5 μ M). For simplicity, untreated tubulin or phosphocellulose purified tubulin will be referred to as PCTb, and NEM-treated tubulin will be referred to as NTb. The rate of elongation at both plus and minus ends decreased with increasing NEM concentration, but more significantly at the minus end. There was no apparent effect of NEM modification on dilution-induced rapid shortening rates. Only one minus end was observed with 0.5 mM NEM treated tubulin (n=50), and no minus ends were ever observed with 1.0 mM NEM treatment, which labeled two cysteines on each α tubulin and β tubulin. The series of video-enhanced DIC images show the inhibition of minus end MT assembly, specifically showing 25 μ M PCTb, and 2:1 PCTb:NTb treated with 0.5 mM NEM. During the elongation phase, the plus end of the axoneme is determined to be the end where tubulin assembly occurs 2-3 times faster. In these images, the plus end is on the right and the minus end on the left.

MT elongation and dilution-induced shortening rates were determined as a function of PCTb:NTb ratio, after determining the concentration of NEM needed to consistently observe effects of inhibition on minus end assembly. Different PCTb:NTb ratios were assembled onto previously existing MT ends (Materials and Methods). Ratios of PCTb:NTb of 2:1 (25 μ M PCTb + 12.5 μ M NTb) and 1:1 (25 μ M PCTb + 25 μ M NTb) were analyzed for the ability to inhibit minus end growth. At the total tubulin concentrations of 37.5 and 50 μ M, plus end elongation rates were lower than those of 25 μ M PCTb alone. Even at these high total tubulin concentrations, minus end assembly was almost completely halted. Elongation at the plus end was inhibited by <20%, while elongation at the minus end was inhibited by >90%. Catastrophes were observed only when the chamber volume was replaced with a 1:1 ratio of PCTb to NTb (one cysteine labeled). Surprisingly, catastrophes were observed at 1:1 ratios of all total tubulin concentrations < 50 μ M. No catastrophes were observed for PCTb concentrations > 12.5 μ M, and here these catastrophes were observed with 25 μ M PCTb (Table 1), suggesting that NTb is acting in a negative manner as if lowering the PCTb

concentration by half. However, all catastrophes were observed under conditions with one cysteine labeled; therefore, the labeling of the second cysteine may act to stabilize the MT conformation in some manner.

After evaluating these ratios, a 4:1 ratio of PCTb:NTb (20 μ M PCTb + 5 μ M NTb) was evaluated for the ability to inhibit minus end growth. Elongation at the plus end was inhibited by <20%, while elongation at the minus end was inhibited by 90%. Shortening rates did not change during the episode of rapid shortening, suggesting no difference in the interactions between NTb and PCTb subunits, when NTb had the opportunity to be incorporated into the MT. A ratio of 4:1 PCTb:NTb was equally effective as ratios of 2:1 or 1:1, indicating that just one labeled tubulin molecule out of 5 was sufficient to inhibit minus-end assembly. No catastrophes were ever observed over a period of thirty minutes.

As just mentioned, 1 NTb subunit per 5 tubulin subunits was sufficient to substantially inhibit minus end assembly. Elongation rates of PCTb were compared to those of 4:1 PCTb:NTb as a function of tubulin concentration. For PCTb, mean elongation rates were plotted as a function of tubulin concentration (8, 10, 12.5, 16.7, 20, 25, 37.5, and 50 μ M). For 4:1, the mean rate of elongation was plotted as the PCTb concentration only (80% of total). The lowest PCTb concentration able to support elongation at both ends was 10 μ M; however, if PCTb is supplemented with NTb to 20% of the final total tubulin concentration to produce a 4:1 ratio, the lowest PCTb concentration able to support elongation at both ends was 16.7 μ M.

It was previously found that the rate of elongation is directly proportional to the free tubulin concentration (Walker et al., 1988). The rate of elongation of the plus end was found to be about twice that of the minus end over the tubulin concentrations examined. Tubulin assembly is dependent on both association (k_2°) and dissociation (k_{-1}°) rate constants. These rate constants can be used to interpret the interactions among tubulin subunits during the elongation phase. The rate constants in Table 2 were calculated from linear regression analysis of the elongation rates previously described. Values for association (k_2°) and dissociation constants (k_{-1}°) during elongation were derived from slope and y-intercept values, respectively, as described by Walker et al., 1988 [1988 #418]. The values of untreated tubulin were compared to those from a 4:1 ratio of PCTb (20 μ M) and 1 mM NTb (5 μ M). NEM-treated tubulin decreased the association rate constant, and also decreased the dissociation rate constant.

Dilution-induced shortening was observed after the chamber volume was replaced with 7-8 chamber volumes of PM buffer. As described for the previous experiment, PCTb dilution-induced shortening rates were compared to those derived from 4:1 PCTb:NTb. Additionally, when the

chamber volume was replaced with NTb alone at 25 μ M (an equimolar ratio to the growing PCTb concentration) resulted in dilution-induced shortening, indicating that NTb cannot maintain existing MTs. The PCTb concentration did not affect the rates of dilution-induced shortening. Compared to MTs assembled from PCTb, those assembled from PCTb:NTb shortened at slower rates.

Analysis of MT Life History Plots

Up to this point, analysis centered on tubulin elongation and dilution-induced shortening rates. In all cases, MTs described are representative of what was consistently observed under specific conditions. In these experiments, 25 μ M PCTb was added for 2-3 minutes and then replaced by 2-3 chamber volumes of 31.25 μ M 4:1 PCTb:NTb (1 cysteine labeled). After about 5 minutes, the solution was replaced with 7-8 chamber volumes of PM buffer to induce MT shortening. MT plus ends continued to elongate at near control rates after being switched from PCTb to 4:1 PCTb:NTb, and shortening rates at the plus end appeared to be similar compared to MTs fully composed of PCTb. In contrast, MT minus ends appeared to either stop elongating or elongated extremely slowly (0.35 ± 0.12 μ m/min) after being switched from PCTb to 4:1 PCTb:NTb. Despite this failure to elongate, minus end MTs did not transition to shortening.

Is it possible that stable minus ends can no longer support tubulin assembly? To determine if these stable minus ends can be elongated, triple perfusion experiments were conducted. Plus and minus MTs assembled in the presence of NTb were compared to MTs fully composed of PCTb. These experiments were identical to those described above, with one additional perfusion of 25 μ M PCTb before the addition of PM buffer to induce shortening. MT plus and stable minus ends returned to control rates after being switched from 4:1 PCTb:NTb to 25 μ M PCTb. Upon dilution-induced shortening, plus end MTs consistently exhibited a pause in shortening upon reaching the last region of the MT that was assembled in the presence of 4:1 PCTb:NTb. The shortening profile of minus end MTs was identical to that of control MTs.

Identification of critical cysteines

To identify reactive cysteines, tubulin was treated with a variety of chemical digestion agents and endoproteinases. Formic acid cleaves between aspartic acid and proline residues. α tubulin has one formic acid cleavage site that should yield two fragments: one 34 kD (8 cysteines) and one 16 kD (4 cysteines). β tubulin has two formic acid cleavage sites that should yield three fragments: one 30 kD (6 cysteines), one 16 kD (1 cysteine), and one 3 kD (1 cysteine). Tubulin was treated with biotin-

NEM, which was found to behave identically to NEM in assembly assays. Biotin-NEM treated tubulin was cleaved with formic acid and samples were analyzed in immunoblots using the anti-biotin antibody. Data was difficult to interpret due to smearing. It could be inferred from the data that the anti-biotin antibody recognized the 34 kD fragment from α tubulin and the 30 kD fragment from β tubulin. However, due to the smearing it was difficult to rule out the two 16 kD bands in each proteins. However, some of the band smearing could have been due to incomplete digestion.

Tubulin was also treated with trypsin and chymotrypsin in partial digestion assays. When used in conditions that allow only partial digestion, trypsin cleaves α tubulin at Arg-339, yielding a 41 kD fragment, and a 14 kD fragment. Under these conditions a 35 kD fragments is sometimes observed due to incomplete digestion. Chymotrypsin cleaves β tubulin at Tyr-281, yielding a 34 kD fragment and a 19 kD fragment. Once again, immunoblotting analysis was difficult to interpret due to smearing. The 41 kD band from biotin-NEM-treated α tubulin was recognized by the anti-biotin antibody. This information narrows down the reactive cysteines in α tubulin to include: 4, 20, 25, 129, 200, 213, 295, and 305. The reactive cysteines in β tubulin could include: 127, 129, 201, 211, and 239. These data suggest that Cys-354 is not labeled by NEM. However, the data do not exclude this cysteine since it has been shown to be reactive with other sulfhydryl-modifying agents, like iodoacetamide. Tubulin peptide samples were sent for sequence analysis by mass spectrometry, in order better identify NEM-reactive cysteines. Unfortunately, further characterization was not successful at this point. The identification of NEM-reactive cysteines is still in progress.

Discussion II

Effects of NEM on Tubulin Assembly

Consistent with previous work (Huitorel, 1988; Hyman et al., 1991), this study showed that NTb is unable to assemble onto axonemes by itself. Additionally, it was observed that NTb is unable to maintain existing MTs by itself. For tubulin dimers, α and β subunits were equally labeled with [3 H]NEM. Maximum labeling is 2 cysteines per subunit, even with 5 mM NEM. This concentration should in theory label all reactive cysteines, being a 200:1 molar excess over tubulin and a 10-fold excess over total cysteines. Unlike what was observed for tubulin dimers, treating taxol-stabilized MT (TMTs) with [3 H]NEM still labeled 2 cysteines on α , but only 1 cysteine on β . One of the reactive cysteines on β may be masked, presumably due to MT assembly. As previously described, Cys-127 and Cys-129 on β tubulin are hidden by tubulin-tubulin interactions that occur

during MT assembly; therefore, Cys-127 or Cys-129 may be labeled by NEM in the unassembled dimer.

Treating tubulin with NEM concentrations of >0.5 mM is sufficient to almost completely inhibit minus end assembly when combined with PCTb, with the final proportion of NTb only 20% of the total. For MTs assembled from PCTb, replacement with the same PCTb concentration supplemented with NTb (final proportion of NTb 20%) inhibited the elongation of MTs by $>90\%$ at the minus end and only $< 20\%$ at the plus end. The rate of shortening is not normally affected by tubulin concentration. At the plus end, the shortening rate was slightly faster overall, while at the minus end, the shortening rate was slightly slower overall. This could imply that NTb was incorporated into the minus end and did not dissociate as quickly as PCTb; therefore, NTb slowed the shortening process slightly.

How fast a MT grows is known to be a function of concentration and the rate constants. The rate constants are derived from the rate of elongation for various concentrations of PCTb. NTb appears to decrease the association rate constant for both ends. The association rate constant for PCTb alone is nearly twice the value of PCTb + NTb. This indicates that NTb slows down the rate at which tubulin subunits assemble onto the MT. Under normal assembly conditions, the dissociation rate constant is a negative value, since it is the value for the off rate (the rate at which tubulin subunits are coming off the MT during the elongation phase). NTb decreases the dissociation rate constant, because it becomes a positive value. This implies that tubulin subunits are coming off slowly or rather not at all, as shown by the stable MT minus end where NTb is believed to be incorporated.

Minus end MTs in the presence of 4:1 PCTb:NTb elongated slowly or not at all (0.30 ± 0.12 $\mu\text{m}/\text{min}$, $n=19$ vs. 2.42 ± 0.47 $\mu\text{m}/\text{min}$, $n=20$) without transition to rapid shortening (catastrophe), suggesting that NTb is acting at the minus end by uncoupling the relationship between catastrophe frequency and elongation rate. Under normal conditions, if PCTb were to assemble at these low rates (0.30 $\mu\text{m}/\text{min}$) there would be a high frequency of catastrophes for those MTs, but this is not observed under conditions where NTb is 20% of the final tubulin concentration. The observed stabilization of minus end MTs is consistent with the absence of MT minus ends in the presence of NTb (Huitorel, 1988; Hyman et al., 1991). Plus end MTs exhibited a pause in dilution-induced shortening during the triple replacement experiments, suggesting that NTb may be incorporated into the MT. It is not clear why this pause only occurs at the interface of the portion of the MT assembled in the presence of NTb, and is not apparent throughout this entire region. Perhaps some NTb is trapped among PCTb subunits that can assemble at a faster rate. Another hypothesis is that since GTP-tubulin disassembles at a slower rate compared to GDP-tubulin, perhaps NTb is always in a GTP form and the β NTb subunits cannot hydrolyze GTP after assembly onto the MT plus end.

Treatment with [³H]NEM indicated that labeling of 1 cysteine on both α and β tubulin was responsible for the observed inhibition of minus end MT assembly. One cysteine on β tubulin was lost when TMTs were treated with [³H]NEM. This cysteine on β tubulin could be Cys-127 or Cys-129 since both are masked during MT assembly. It is interesting to note that the GTP exchangeable binding site in β tubulin is exposed in the dimer and could be labeled by NEM, but this site is buried in MT assembly (Nogales et al., 1998). Other possible cysteines include Cys-239, Cys-211, and Cys-201. These three cysteines interact with the guanine nucleotide in β tubulin and are all exposed on helices 6 and 7 as shown in the crystal structure (Nogales et al., 1998). The cysteines on α tubulin have not yet been well characterized concerning reactivities with sulfhydryl reacting agents. Cys-213 and Cys-295 in α tubulin are located close to the GTP binding pocket; therefore, they could act in stabilizing the minus end through possible interactions with GTP. In fact, Cys-295 has been crosslinked to the guanine nucleotide in α tubulin (Bai et al., 1989).

Table 1. Catastrophe and Rescue Frequencies

No catastrophes were ever observed for PCTb concentrations $\geq 12.5 \mu\text{M}$.

Catastrophes were observed when the chamber volume was replaced with a 1:1 ratio of PCTb:NTb (one cysteine labeled).

Tubulin Concentration or Condition	Frequency of Catastrophe/ Rescue		Time (seconds)	
	Plus	Minus	Plus	Minus
50 μM	0	0	2190	2400
37.5 μM	0	0	2280	2640
25 μM	0	0	2640	3840
25 μM DTT-depleted Tb	0	0	3300	3840
20 μM	0	0	3630	3840
16.7 μM	0	0	3980	4020
12.5 μM	0	0	4320	4380
10 μM	0	C: 0.0024 R: 0.0010	2800	2940
8 μM	C: 0.0026	None	1920	None
4:1 replacement (1 Cys)	0	0	3780	5160
4:1 replacement (2 Cys)	0	0	3840	5280
2:1 replacement (1 Cys)	0	0	4860	5400
2:1 replacement (2 Cys)	0	0	4800	5460
1:1 replacement (1 Cys)	0	C: 0.0025 R: 0.0019	4200	3600
1:1 replacement (2 Cys)	0	0	4200	4740

Table 2. Association and Dissociation Rate Constants

Slope, x-intercept, and y-intercept values were derived from the regression lines plotted in Figure 25.

Constants	PCTb		4:1 PCTb	
	(+)	(-)	(+)	(-)
Slope ($\mu\text{m}/\mu\text{M min}$)	0.26	0.11	0.17	0.03
k_2^e ($\mu\text{M}^{-1}\text{s}^{-1}$)	7.2	3.1	4.7	0.68
x-intercept (μM)	3.0	1.9	-----	-----
y-intercept ($\mu\text{m}/\text{min}$)	-0.79	-0.22	0.49	0.48
k_{-1}^e (s^{-1})	-21.5	-6.0	-----	-----

Conclusion

Chemical modification is an important tool for probing structure-function relationships of reactive amino acids. In this project, the sulfhydryl-reactive agent N-ethylmaleimide (NEM) was used to modify both MT-motor proteins and tubulin. This was done to gain a better understanding of what is responsible for MT-motor protein interactions, and also to gain a better understanding into the mechanism of NEM-inhibition on minus end assembly.

Chemical modification has been an important resource throughout the process of identification and characterization of motor proteins. NEM-treatment of kinesin has shown that the sulfhydryl groups in kinesin are not readily exposed, and suggests that those in the tail are more reactive than those in the motor or stalk. Interestingly, the plus end-directed motor kinesin must be treated with a relatively high dose of NEM (1-10 mM) to observe any effect. In comparison, the minus end-directed MT motors dynein and Ncd exert similar effects with low NEM treatment (<1 mM). A reactive cysteine in Ncd may be located just outside the neck region, which is the region now believed to be associated with determination of directionality. Unfortunately, the function of this cysteine is not clear at this time.

NEM-treated tubulin has long been used as a tool for characterizing MT-motor proteins. Until now, it was not clear what was acting to inhibit minus end assembly. It has now been observed that NEM-treatment results in stabilizing the minus end only and not the plus end. However this is only a temporary stabilization and is not a permanent cap of NEM-tubulin. Dynamic instability is not completely understood, and analyzing what is happening at the minus end is a start to this process. NEM-tubulin could become an important resource into understanding what is happening at the minus end where α tubulin is exposed. Another type of tubulin found in nearly all eukaryotic cells is γ tubulin, which binds to MT minus ends, and is involved in nucleating MTs. At this time, γ tubulin is not well characterized and NEM-tubulin could be used as a tool in the future for analyzing γ tubulin. Cysteine residues in tubulin are of great significance due to their interactions with anti-mitotic drugs. Further, multiple forms, or isoforms, of tubulin exist. While the reasons for having multiple forms are not well understood, the isoforms are believed to have evolved as necessary for living in varying conditions. Particular isoforms of tubulin are more common in certain organs or under certain conditions. For example, $\alpha\beta_{III}$ is most common in tumors. Tubulin isoforms can also differ in their cysteine content, $\alpha\beta_{III}$ share several cysteines with the other isoforms but not all. Any knowledge gained concerning the reactivity of cysteines in tubulin and MT-motor proteins may be beneficial for drug-targeting and drug-specificity in treating cancer, and perhaps other-related health problems like, neurological defects, infertility, and birth defects.

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