A New Look at the Microtubule Binding Patterns of Dimeric Kinesins

Andreas Hoenger1*, Manfred Thormählen2, Ruben Diaz-Avalos3
Monika Doerhoefer1, Kenneth N. Goldie1, Jens Müller2
and Eckhard Mandelkow2

1European Molecular Biology Laboratory, Structure Programme, Meyerhofstrasse 1
D-69117 Heidelberg, Germany
2Max Planck Unit for Structural Biology, DESY
Notkestrasse 85, D-22607 Hamburg, Germany
3Institute of Molecular Biophysics, Florida State University, Tallahassee FL 32306, USA

The interactions of monomeric and dimeric kinesin and ncd constructs with microtubules have been investigated using cryo-electron microscopy (cryo-EM) and several biochemical methods. There is a good consensus on the structure of dimeric ncd when bound to a tubulin dimer showing one head attached directly to tubulin, and the second head tethered to the first. However, the 3D maps of dimeric kinesin motor domains are still quite controversial and leave room for different interpretations. Here we reinvestigated the microtubule binding patterns of dimeric kinesins by cryo-EM and digital 3D reconstruction under different nucleotide conditions and different motor:tubulin ratios, and determined the molecular mass of motor-tubulin complexes by STEM. Both methods revealed complementary results. We found that the ratio of bound kinesin motor-heads to $\alpha\beta$-tubulin dimers was never reaching above 1.5 irrespective of the initial mixing ratios. It appears that each kinesin dimer occupies two microtubule-binding sites, provided that there is a free one nearby. Thus the appearances of different image reconstructions can be explained by non-specific excess binding of motor heads. Consequently, the use of different apparent density distributions for docking the X-ray structures onto the microtubule surface leads to different and mutually exclusive models. We propose that in conditions of stoichiometric binding the two heads of a kinesin dimer separate and bind to different tubulin subunits. This is in contrast to ncd where the two heads remain tightly attached on the microtubule surface. Using dimeric kinesin molecules crosslinked in their neck domain we also found that they stabilize protofilaments axially, but not laterally, which is a strong indication that the two heads of the dimers bind along one protofilament, rather than laterally bridging two protofilaments. A molecular walking model based on these results summarizes our conclusions and illustrates the implications of symmetry for such models.

© 2000 Academic Press

Keywords: kinesin; microtubules; cryo-electron microscopy; helical 3D image reconstructions; kinesin movement

Introduction

The cellular, biochemical and structural information on the kinesin superfamily has reached a level which allows to draw a rather detailed picture of their functions and properties (for reviews see: Hirokawa, 1998; Bloom & Goldstein, 1998; Mandelkow & Johnson, 1998; Mandelkow & Hoenger, 1999; Sheetz, 1999; Endow, 1999). The interaction of kinesin-like motor domains with microtubules has been extensively studied by several groups using electron microscopy and computational 3D analysis (Hirose et al., 1995, 1996, 1998, 1999; Hoenger et al., 1995, 1998; Hoenger & Milligan, 1997; Kikkawa et al., 1995; Arnal et al., 1996, Arnal & Wade, 1998; Sosa et al., 1997a,b; Rice et al., 1999; Kikkawa et al., 2000). The atomic structures of monomeric kinesin and ncd motor domains have been solved by X-ray crystallogra-
phy (Kull et al., 1996; Sabin et al., 1996; Sack et al., 1997). More recent X-ray structures of dimeric motor constructs (Kozielksi et al., 1997; Sabin et al., 1998) revealed further insight into the motor neck regions and the dimerization properties of the z-helical coiled-coil within the stalk. Finally, a 3D map of the zتب-tubulin heterodimer at near atomic resolution has been solved by electron crystallography from zinc-induced tubulin sheets (Nogales et al., 1998). These data are the basis for molecular docking experiments, which combine intermediate resolution data from 3D cryo-electron microscopy with high-resolution data from electron or X-ray crystallography. This now enables structural interpretations of large macromolecular assemblies such as intact microtubules (Nogales et al., 1999) and microtubule-motor complexes to near-atomic detail (Sosa et al., 1997a; Hoenger et al., 1998; Kozielksi et al., 1998; Hirose et al., 1999; Rice et al., 1999; Kikkawa et al., 2000).

Cryo-electron microscopy 3D reconstructions revealed a consensus view on the overall arrangement of dimeric ncd motor domains complexed with microtubules (Sosa et al., 1997a; Hirose et al., 1998). Ncd dimers interact with tubulin through one of their heads with the second ones protruding outward. Slight conformational changes upon nucleotide exchange have been reported within the unattached heads (Hirose et al., 1998). However, in the case of dimeric kinesin the situation is much more confusing (Hirose et al., 1996 and 1999; Arnal et al., 1996, Arnal & Wade, 1998; Hoenger et al., 1998; see also Figure 1). There is a general agreement on the densities related to one strongly bound head domain per zتب-tubulin heterodimer. However, significant differences exist in the interpretations of the volumes that relate to the second head of the dimer (Figure 1; compare Hoenger et al., 1998 with Hirose et al., 1996 and 1999 and Kozielksi et al., 1998). Some maps were interpreted to show loosely bound heads, analogous to the second head of ncd, but containing only a fraction of the expected mass. This lack of mass was interpreted as the result of an intrinsic flexibility of these so called “loosely bound” heads at this position, which, nevertheless, was considered to reflect a relevant conformational state of the kinesin dimer (Hirose et al., 1996, 1999; Arnal & Wade, 1998). The “loose” head had a different orientation than that of ncd, which was interpreted to reflect the different directionalities of the two motors (see also Endow, 1999).

Here we used cryo-electron microscopy (cryo-EM) and 3D image reconstruction, scanning transmission electron microscopy (STEM) mass determinations and molecular modeling to clarify the properties of dimeric kinesin-microtubule complexes. Our findings address two key-issues which are different in our interpretation from earlier ones: (1) How accurate are the current 3D reconstructions of microtubules decorated with dimeric kinesin under conditions where both heads potentially may interact with the microtubule surface at the same time? (2) How well defined and unique are the conformational states of apparently loosely-bound (supposed not to contact the microtubule surface) heads of kinesin dimers under near-physiological conditions?

Given that the apparent electron densities of kinesin dimers on microtubules revealed inconsistent results among different groups, the attempts to correlate them with the X-ray structure led to different docking models. Two of these models show one tightly and one loosely bound motor head per zتب-tubulin dimer, however, both reveal a different orientation (Kozielksi et al., 1998; Hirose et al., 1999; Figure 1(b) and (c)). In addition, our previous results showed a separation of heads and docking onto two different β-tubulin subunits (Hoenger et al., 1998; Figure 1(a)) a similar model was implied by kinetic arguments (Romberg et al., 1998). Here, we show additional evidence that the two heads bind along one protofilament (Figure 5). We now believe that, in contrast to dimeric ncd (Sosa et al., 1997a) the appearance of the loosely bound kinesin head does not reflect a physiologically relevant state. Our results indicate that the conformations of dimeric kinesins on helically averaged 3D maps are induced by an oversaturation of available microtubule binding sites (see Figure 6). The additional mass takes the appearance of loosely tethered but periodic heads as a result of the helical averaging procedure. Finally, by proposing our walking model we argue that a hand-over-hand mechanism cannot be achieved without the involvement of at least two alternative pathways on how the trailing motor domain switches into the leading position (see also Cross, 1995; Howard, 1996; Figure 9).

**Results**

**3D maps of kinesin-microtubule complexes**

We calculated a series of 3D maps of microtubules complexed with monomeric and dimeric kinesins from rat, squid, *Drosophila* and *Neurospora* (Figures 2 and 3) either in the presence of AMP-PNP or in the absence of ATP or ADP (after apyrase treatment). We used this broad approach to screen for a potential species-related bias in our maps. The 3D reconstructions represent helical averages from several (approximately 10-20 each) selected 15-protofilament microtubules (Figure 2) at a resolution of approximately 25 Å. The individual maps consist of ~17,000 to 30,000 asymmetric units each. Data processing was carried out according to published procedures (Sosa et al., 1997a; Hoenger et al., 1998) using PHOELIX (Whittaker et al., 1995). A typical phase/amplitude plot of an averaged map is shown in Figure 2(c). Phase and amplitude data were collected up to the approximate position of the first zero-node of the contrast transfer function (at approximately 20-25 Å resolution).
Tightly bound kinesin head domains do not undergo large-scale conformational and positional changes during ATP hydrolysis

Irrespective of their origins, all single and double-headed kinesin constructs used here revealed similar structural features when complexed to microtubules. All maps showed a strongly bound head at the same microtubule-binding site throughout all species and nucleotide states (see Figures 3 and 4). In agreement with other reconstructions (Arnal & Wade, 1998; Hirose et al., 1999; Hoenger et al., 1998), the motors shown here are elongated ellipsoids with their long axis parallel to the protofilament axis (Figures 3 and 4). Their features are very reproducible among all different nucleotide states and decoration stoichiometries. They all have a protrusion at the plus-end pointing slightly to the right. The motor becomes flatter and slightly wider at its base in minus-end direction. The inside of the microtubule wall represents a good control for the accuracy and reproducibility of the maps (Figure 4 arrows). The conformational changes on the level of secondary structural elements within the tightly bound heads among different nucleotide states which were visualized very recently by using gold-labeling of the kinesin neck regions (Rice et al., 1999) are barely detectable at the resolution of 25 Å without such labels. However, in agreement with their results we now can exclude the existence of large-scale conformational changes in motor-tubulin interaction during ATP hydrolysis. The reproducible features on the inside of the microtubules (see Figure 4, blue densities) and their consistent position relative to the motors on the outside argue against the possibility that the motors switch from one tubulin binding site to the other (e.g. from β- to α-tubulin) between different nucleotide states.

Decoration with dimeric kinesin shows only a fractional loosely bound head per tubulin heterodimer

In contrast to the tightly bound heads, some nucleotide dependent conformational changes were apparent within the second heads of the dimer, here referred to as loosely bound heads. The strongest evidence for loosely bound heads was found with dimeric Drosophila kinesin, and this in particular in the absence of nucleotide (Figures 3(b) and 4(a)). The corresponding volume represents ~50% of that of a tightly bound head indicating partial absence and/or disorder. Its flattened, disc-like shape is indicative for disorder, but as shown below, STEM mass determinations clearly exclude disorder as the sole source of reduced volumes. In the presence of AMP-PNP, Drosophila kinesin showed even smaller traces of loosely bound heads with a residual mass of approximately 10-20% compared to a tightly bound head. Again, its shape is very flat with sharp edges (Figure 4(b)), which suggests a disordered position combined with partial occupancy. The maps do not show unambiguously to which side the stronger connections really extend. It is possible that the loosely bound heads interact side-ways with the adjacent tightly bound head in a similar way as observed here with monomeric squid kinesin (i.e. to the left in Figure 3(f), (g), Figure 4(e)). Dimeric rat kinesin revealed the same results as published earlier (Hoenger et al., 1998). Our 3D reconstructions using rat kinesin constructs did not detect any mass related to loosely bound
heads at all (Figures 3(c), (d), (e) and 4(d)). In summary, helical averaging and 3D reconstruction revealed stoichiometries between approximately 1 (rat) and 1.5 (Drosophila) kinesin head domains per αβ-tubulin heterodimer. None of the constructs ever showed a stoichiometry of two heads per tubulin dimer e.g. as found previously with dimeric ncd constructs (Sosa et al., 1997a; Hirose et al., 1998). These results were obtained at initial kinesin-dimer to tubulin-dimer ratios of 2:1 which allows an equi- or supersaturation of binding sites (see below). These values seem somewhat lower than the figures from STEM determinations which may be due to the averaging procedure which only picks up mass which follows the assumed helical regularity, while STEM measurements detect everything present.

STEM mass determination of microtubule-motor complexes revealed a binding pattern consistent with the 3D reconstructions

STEM mass determinations were used to check the results from the 3D reconstructions of motor-microtubule complexes, and to make a clear distinction of partial occupancy from simple dislocation of the loosely bound heads. Unlike 3D image reconstruction, which relies on data averaging, this approach is insensitive to minor dislocations of mass, but detects an apparent partial occupancy with high accuracy. The results are summarized in Table 1. The measured mass of undecorated microtubules was used as a calibration and matched well the expected value, in agreement with previous measurements.
The samples were prepared in the same way as for cryo-electron microscopy. No chemical fixation was applied. The initial molecular ratios of kinesin- to tubulin-dimers during the decoration process were kept at 2:1. The molecular masses of the kinesin-microtubule complexes correspond to stoichiometries of one motor head per tubulin dimer for monomeric Neurospora- and rat-kinesin constructs. We measured approximately 1.5 motor heads for dimeric Drosophila kinesin DHK392 under all nucleotide states. The values were slightly lower

<table>
<thead>
<tr>
<th>STEM mass determinations of motor-tubulin complexes</th>
<th>Monomeric constructs</th>
<th>Dimeric constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αβ-tubulin</td>
<td>nK343</td>
</tr>
<tr>
<td>Expected mass (kDa/nm²)</td>
<td>5.50</td>
<td>7.39</td>
</tr>
<tr>
<td>Measured mass (kDa/nm²)</td>
<td>(+/− 0.67)</td>
<td>(+/− 0.70)</td>
</tr>
<tr>
<td>n</td>
<td>430</td>
<td>181</td>
</tr>
<tr>
<td>Heads per αβ-dimer</td>
<td>~0.95</td>
<td>~1.5</td>
</tr>
</tbody>
</table>

The measurements made clear that the stoichiometry of dimeric kinesin constructs only reached a value of approximately 1.5 motor heads per αβ-tubulin dimer. These values may even appear higher than what would be expected from the 3D image reconstructions. However, one has to keep in mind that a combination of partial absence and dislocation strongly reduces the appearance of regular volumes in averaged reconstructions, but keep the overall mass at the levels measured here. Accordingly, sK338 indicates a well-defined location but partial absence, while the numbers found for rK379-A339C indicate both partial absence combined with dislocation.
for dimeric rat kinesin (1.3). Dimeric ncd revealed a ratio of 2.0 heads tubulin dimer. Finally, the ratio obtained with monomeric squid kinesin was at 1.5 heads per tubulin dimer, and thereby confirmed the image reconstruction results shown in Figures 3 and 4 which show a small mass related to a loosely bound head. Generally the mass-measurements agree well with the observations from cryo-EM. We could not find any decoration, which reached a mass corresponding to a stoichiometry of two kinesin heads per tubulin heterodimer. The unexpected appearance of loosely bound heads in reconstructions of monomeric squid kinesin sK338 (see Figure 4(e)) was confirmed as well. The results here also compare well to our STEM measurements on dimeric ncd, which was previously found to bind to microtubules with a stoichiometry of two heads per αβ-tubulin dimer (Sosa et al., 1997a; Hirose et al., 1998). The observed stoichiometries are consistent with a supersaturated microtubule lattice (1 tightly bound motor head per β-tubulin, and in 30-50% of the sites a loosely bound head that was unable to find a proper docking position), but they are not consistent with models of one tightly, one loosely bound head for every β-tubulin (implied by Arnal et al., 1996 and Hirose et al., 1996 and 1999).

**Figure 4.** This Figure illustrates the three different ways loosely bound heads may appear on helical reconstructions from dimeric kinesin constructs. As in Figure 3 tightly bound heads are yellow and loosely bound ones are red. The positions of tightly and loosely bound heads on the end-on projections are marked with yellow and red arrows respectively. The position of the αβ-tubulin dimer is indicated in (a). Its features are quite reproducible and their positioning relative to the tightly bound motors indicate that the binding positions of motors do not change among different species and nucleotide states. The shapes of tightly bound heads also appear very reproducible among all reconstructions. (a) Dimeric *Drosophila* kinesin DHK392 in a no-nucleotide state. This state shows the strongest signal of a loosely bound head domain. Here the major connections appear to extend sideways to heads on adjacent protofilaments. Even less mass remains on reconstructions of DHK392 in an AMP-PNP state shown in (b). An overload of the microtubule binding sites by eight times more dimers than microtubule-binding sites during decoration increases the residual mass to some extent (c). However, it still remains well below the mass of a full head. Its shape is very indicative for an ill-defined position. The additional mass is clearly visible on the end-on projections (red arrows; bottom row). (d) No loosely bound head is visible neither on the 3D rendered volume nor on the end-on projection of dimeric rat kinesin. (e) Monomeric squid kinesin, on the other hand, appears to dimerize via a different head to head interaction, which is not mediated by a coiled-coil.

**Binding properties of kinesin dimers influenced by the decoration kinetics**

Our possible explanation for the appearance of additional loosely bound heads is the overloading of the microtubule lattice so that some heads cannot access a proper binding site and instead protrude beyond the properly bound heads. A rapid saturation of microtubule binding sites should lower the chance for a dimeric molecule to find two free binding sites nearby as illustrated in Figure 6. Thus an increasing number of head domains will be forced into a loosely bound position. After periodic averaging, this would create additional densities with a mass less than that of a full head. This effect, however, should depend on the decoration conditions, and in particular on the initial mixing ratios used between kinesin dimers.
to tubulin dimers during the decoration process. We could demonstrate this effect here for dimeric *Drosophila* kinesin DHK392 (Figures 3(a), 4(b) and (c)). We observed that raising the stoichiometry of dimeric kinesin heads to tubulin-dimers from 2:1 to 8:1 increased the residual mass of the loosely bound head by about a factor of two (Figure 4(b)). Their relative masses, however, still remained below a value expected for a well-defined conformational state at 100% occupancy (compare Figure 4(b) and (c)). We therefore conclude that overloading the microtubule lattice with dimeric motor domains can modulate the appearance of loosely bound second heads.

**Monomeric squid kinesin motors interact with microtubule-bound heads through a head-to-head interaction, which is not mediated by the coiled-coil**

The monomeric squid construct sK338 offered a surprise in that it behaved similarly to the dimeric *Drosophila* constructs (Figures 3(f), (g), 4(e)). Here the 3D reconstructions of microtubules complexed with this head domain revealed the presence of a second loosely attached head even though this construct is monomeric in solution (Marx et al., 1998). This interaction cannot be mediated by coiled-coil interactions of the neck since the construct ends at position 338 right at the start of the neck helix (MARX et al., 1998). Similarly to dimeric *Drosophila* kinesin, the reduced relative mass indicates only partial occupancy. The globular shape, however, is typical for a relatively stable configuration, once a head is there. This phenomenon has been observed in both, AMP-PNP and no-nucleotide states, but it was more pronounced in the AMP-PNP state. According to our previous docking results (Hoenger et al., 1998; Figure 7(a)) this additional density locates close to loop 2 of the tightly bound head. Monomeric rat and *Neurospora* kinesins revealed structures as expected from previous results (Figure 3(h) and (i)). Dimeric ncd from Sosa et al. (1997a) is shown for comparisons (Figure 3(k)).
A dimeric rat kinesin, which is crosslinked at the beginning of its neck region, stabilizes microtubules axially but not laterally

An interesting behavior was observed with a mutant rat kinesin construct, based on rK379, here referred to as rK379-A339C. In this mutant alanine 339 has been exchanged with a cysteine. Its position is at the very beginning of the neck-helix and allows crosslinking of the dimeric state by a disulfide bridge directly behind the head domains. We expected this additional stabilization of the dimer contact to prevent a (presumed) dissociation of the neck-helices e.g. as modeled in Figure 8 and, thus, increasing the frequency of loosely bound heads. Consistent with this, the cross-linked mutant binds to microtubules but shows differences in its microtubule-stimulated ATPase activity (Table 2). The $K_{(0.5 \text{ MT})}$ values of the oxidized form of this mutant were found to be 16.4 μM compared to 1.24 μM under reducing conditions, and 0.42 μM for the wild-type construct rK379. However, in contrast to our expectations the 3D maps strongly resembled the maps obtained with wild-type rK379 with a stoichiometry of approximately one head per tubulin dimer. A more frequent occurrence of loosely bound heads was not observed, and STEM measurements revealed motor:tubulin-dimer stoichiometries of 1.5, comparable to wild-type kinesin-tubulin complexes. Nevertheless, this mutant showed an interesting effect on the stability of protofilaments in axial direction (Figure 5). Similar effects have been observed with any other constructs as well but never at such a magnitude. Small bundles of protofilaments remained assembled over several hundred μtubulin heterodimers forming gently curved structures (Figure 5), with high radii of curvature (~100 – 150 nm). These curves are much wider than those of tubulin rings (~20 nm) and are reminiscent of tubulin hoops or gently curved groups of protofilaments of growing microtubules (Mandelkow et al., 1977 and 1986; Chretien et al., 1996). The motors are always found on the inner side of the bent protofilaments. Occasionally, microtubules can also be found which disassemble laterally within an otherwise intact tube; see Figure 5. These features are a strong indication that the two motor domains of a kinesin dimer bind to the same protofilament in axial direction. In our model as shown in Figure 8(d), the beginning of the coiled-coil neck is opened by about 1.2 nm. It could be possible that preventing the opening of that region may cause some strain to the tubulin protofilament, which in turn might cause some elastic deformation within the kinesin dimer.

### Discussion

3D maps and STEM indicate binding properties of dimeric kinesin which support a basic stoichiometry of one motor domain per tubulin heterodimer

We calculated 3D maps of microtubules decorated with monomeric squid, rat and *Neurospora* kinesin constructs, and dimeric rat and *Drosophila* kinesin. The maps obtained of dimeric kinesins differ significantly from maps of dimeric ncd (Sosa et al., 1997a; Hirose et al., 1998). Our 3D maps of
monomeric and dimeric rat kinesin in the presence of AMP-PNP and in the absence of nucleotide showed no apparent differences to each other (Figures 3(c), (d), (h) and 4(d)). This indicates that in both cases one head (each head of the dimer) interacts with one tubulin dimer. Therefore, in the case of the dimeric rat constructs we conclude that both heads bind to two adjacent binding sites on the microtubule surface according to the situation illustrated in Figures 6(c) and 8(d). This is in sharp contrast to the binding properties of ncd where a complete dimer interacts with one tubulin dimer (Figure 3(k); Sosa et al., 1997a). The situation is somewhat different with dimeric Drosophila kinesin constructs. Here we detect volumes corresponding to motor domains, which are tethered to the tightly bound domains, but do not directly interact with the microtubule surface. The corresponding volumes and the molecular masses determined by STEM (Table 1), however, indicate that these volumes only represent up to 50% of a full head. We conclude here that this is an effect of overloading the microtubule surface as illustrated in Figure 6(b). Accordingly, the periodic appearance of these volumes is the result of regular helical averaging over several conformational states.

The appearance of loosely bound heads on the microtubule surface depends on the saturation conditions during the formation of motor-microtubule complexes

We demonstrated that the appearance of loosely bound heads and their structural stability could be regulated to some extent by increasing the initial stoichiometrical ratio of dimeric motors to tubulin heterodimers during decoration (Figure 4(b) and (c)). This is a direct effect of decreasing the chance for the “slower” one of the dimer heads to find an empty binding site next to the first one. Consequently, if there are no free binding sites available, this head has to remain in its “uncomfortable” position somewhere above the surface of a motor-saturated microtubule. The conclusion is that apparent conformations of the second head, (which are most apparent under over-saturating conditions) do not constitute a physiological state but rather are the consequences of sterical crowding during the decoration process. We propose that for the particular nucleotide states tested (AMP-PNP state and no-nucleotide state), both heads of kinesin show a potential to bind to the microtubule surface at the same time. Thus, all heads on a fully decorated microtubule which remain unattached, indicate a conformation in which they “stumble” over an already occupied binding site (see Figure 6). We found the largest volumes of loosely bound heads with Drosophila kinesin in the absence of nucleotide. Here one could argue that, in accordance to the new data of Rice et al. (1999), the stabilization of the neck region in an AMP-PNP state directs the second head towards the next adjacent binding site. This could promote a double-binding conformation as suggested in Figure 8(d) and, thus, result in a lower mass at the position of loosely bound heads. The maps obtained with monomeric squid kinesin (Figures 3 and 4(d)) are indicative for a potential stabilization of unattached heads on the microtubule surface which may be mediated by
other head-to-head interactions. These interactions may play a role in the walking process, but the details remain to be further investigated.

**Dimers with a crosslinked neck region stabilize protofilaments in axial direction and enforce a steady curvature on small bundles and single protofilaments**

Strong evidences for a functional involvement of the coiled-coil in kinesin motion come from a mutant kinesin, which contains an alanine-to-cysteine mutation at position 339 at the very beginning of α7 (see Figures 7 and 8). These cysteines can be covalently crosslinked by a disulfide bridge and thereby prevent the neck region from dissociation. The maps obtained with this mutant do not show an increased occurrence of loosely bound heads the way we have expected it in the first place. Instead, the overall structure of partially dissociated microtubules as presented in Figure 5 is indicative for a dimeric kinesin, which binds with both heads to a protofilament in an axial orientation. The question here is: how compatible is this

---

**Figure 8.** Proposed conformational states of dimeric kinesins during their interaction with tubulin protofilaments. Our results from cryo-EM indicate that the kinesin conformation as it was found in the 3D crystal packing (c) of ADP-kinesin (Kozielski et al., 1997) changes substantially upon binding to microtubules, both, in the presence of AMP-PNP, or in the absence of nucleotides (apyrase treatment). Based on our previous observation of a 16-nm arrangement of dimeric kinesins on the surface of tubulin sheets we suggest a model as shown on (d) where both heads of the kinesin dimer bind to two consecutive tubulin heterodimers along one protofilament. Thereby the neck region of (at least) one of the heads (here: the right one) has to undergo substantial conformational changes. These changes, however, are hard to resolve with cryo-EM. We modeled the conformation shown here by bending the neck at three different "hinges". They were placed (1) at the end of helix a6 at position 325, (2) the beginning of helix α7 (pos. 339) and (3) at position 355. The last hinge was positioned at the beginning of the segment II of the neck coiled coil (Tripet et al., 1997; Thormählen et al., 1998b) which marks the start of a strong coiled-coil interaction. A closer look at the neck region and the beginning of (a) the α-helical coiled-coil of ncd and (b) kinesin reveals significant structural features, which may account for their different conformations when bound to microtubules. The leucine zipper of ncd reaches to the very end of the neck helix and the X-ray structure even shows some interaction with the head domains itself (Sablin et al., 1998; left inset). On the other hand, the kinesin neck shows a different composition containing mainly charged amino acid residues. The atomic structure (Kozielski et al., 1997), however, does not reveal any strong attracting forces among this region, rendering this part of helix α7 perfectly stable in solution (see Sack et al., 1997).
with the assumption that the neck dissociates during walking? It appears that the crosslinked mutant dimer is capable of spanning two binding sites but that partial melting of the N-terminal region of helix α7 remains essential for walking and microtubule-activated ATP hydrolysis, which is not possible for the mutated dimer. This is supported by two observations: (1) In its oxidized form this mutant shows a strongly increased $K_{0.5 \text{ MT}}$ for microtubules (16.4 μM instead of 1.24 μM, see Table 2). Since in our decoration experiments the microtubule binding properties of this motor appear unchanged, this value indicates that this mutation allows binding to microtubules but interferes with the processivity. (2) The mutant kinesin significantly stabilizes protofilaments axially (Figure 5). However, it enforces a curvature on single protofilaments. It appears that this represents the most relaxed conformation of these motor-tubulin complexes since it slightly shortens the axial repeat between the dimer heads. Note that the motors are always located to the inside of the curvatures, corresponding to the typical inside-out curvature of protofilaments found in several polymorphic assembly forms of tubulin. Such a stabilizing effect on small bundles and single protofilaments has sometimes been observed with other dimeric constructs as well but to a much lesser extent.

Most important, however, is the observation that microtubules complexed with this oxidized mutant strongly indicate that the two heads of kinesin actually do bind along one protofilament and do not connect between two adjacent ones. The stabilization of protofilaments is clearly in axial direction while allowing the microtubule wall to splay open laterally (see Figure 5).

Motor-microtubule interactions

Figure 7 shows a model of the interaction between the kinesin motor domain and the β-tubulin dimer which is based on our docking attempts published in Hoenger et al. (1998) and those of Nogales et al. (1998) for the tubulin dimer. Based on biochemical and structural considerations this model reveals a motor-tubulin interaction between the predominantly positively charged elements of the kinesin motor domain (α4, α5, β5α/b) and the highly negatively charged helix 12 of β-tubulin (Figure 7). For example arginine 280 (Arg278 in human kinesin), which has been found to be crucial for the motor-tubulin interaction (Woehlke et al., 1997) locates in close proximity to several negative charges of helix 12 in β-tubulin. As illustrated in Figure 1 our kinesin-docking model differs significantly from the ones presented by Kozielski et al. (1998) and Hirose et al. (1999). Based on our results one has to assume a different dimer configuration upon microtubule binding than in solution (Marx et al., 1998). While X-ray crystallography revealed the kinesin dimer with ADP bound to both heads simultaneously this is most likely not the case anymore upon binding to the microtubule surface. This assumption is in good agreement with alternating head catalysis (Hackney 1994; Hancock & Howard, 1999).

Walking model

In order to get a more detailed picture of how a molecular motor such as kinesin may move along a microtubule, we modeled the situation as expected for a hand-over-hand mechanism. As described in Materials and Methods, this was based on our recent attempts to dock atomic resolution structures of individual components into the EM-derived 3D scaffolds of large complexes (Hoenger et al., 1998; Nogales et al., 1999). The results have to be seen as a conceptual walking model illustrating the sterical and geometrical requirements. Our data do not yet allow an accurate determination of the pathways in molecular detail, which underlies kinesin processivity. Nevertheless, it demonstrates the concept and the implications, which have to be considered for model building in general and for the reconstruction of a plausible walking process. From the 3D EM data alone we cannot draw any conclusions on how nucleotide hydrolysis affects the structural states of the motor core and neck region. However, it seems plausible that some nucleotide states promote locking the neck region in one or the other position, e.g. by modulating its interactions between the N terminus and the "gearbox" residues (Henningsten & Schliwa, 1997; Case et al., 1997; Endow & Waligora, 1998).

Hand-over-hand walking requires two mechanically different steps

The two heads of a homodimeric kinesin are not mirrored structures such as human feet, but are two identical units with identical geometrical and biochemical properties. Consequently one of the major implications for hand over hand walking is that processive movement cannot be achieved with a single stepping mechanism and identical conformational pathways for both motors (Figure 9). This would wind up the stalk and either rotate the cargo or produce torsional stress to motor and stalk. These symmetry constraints are rather universal, and do not apply to hand-over-hand mechanisms but generally to all types of stepping models (see also, Howard, 1996). The question remains whether the heads are constantly switching between two alternative states or remain in completely separate states once the initial microtubule contact occurred. Consequently, this leaves two conceptionally different options: (1) During processive movement one of the heads continuously maintains a different conformation from the other one (e.g. as for a "limping" model or for a parallel walk along two adjacent protofilaments). (2) Alternatively, the trailing head has to be capable of passing the leading one in at least two
very different independent pathways (as in a hand-over-hand model; Figure 9). This means that the heads must be able to pass the stalk more or less alternately on the left and the right side otherwise the stalk would experience significant torsional stress after a few steps (see Figure 9). This implication is of general nature, and is nothing more than a direct consequence of the symmetry in dimeric kinesin. It is also completely independent from any particular configuration of the head domains on the microtubule surface e.g. as the ones presented in Figure 1.

The X-ray structure of kinesin indicates a weak coiled-coil interaction at the beginning of the neck helix

The atomic structure in helix α7 of kinesin (Figure 8(b)) supports our idea of a partial melting of the coiled-coil during walking (Tripet et al., 1997; Hoenger et al., 1998; Mandelkow & Hoenger, 1999; Figures 8(d) and 9). The kinesin neck shows strong hydrophobic leucine zipper-like interactions from Leu356 towards the C terminus (Figure 7(b)). The neck helix contains many charged side-chains on the N-terminal side of this residue, which do not appear to mediate a strong dimer attraction (Thormählen et al., 1998b; Morii et al., 1997). Unlike the ncd motor domains (Sabin et al., 1998), the kinesin heads do not seem to interact with their neck helices (Thormählen et al., 1998b). These structural differences permit a reasonable explanation for the observed differences on 3D maps of ncd- and kinesin-microtubule complexes derived from cryo-EM.

The hand-over-hand model (as well as most other of the models) requires substantial structural changes within the kinesin core and neck regions. Here, we attempted to keep the kinesin core structure as constant as possible since there is yet little data available on conformational changes in that area (Müller et al., 1999). As mentioned above, there is so far no indication that ATP hydrolysis causes large-scale changes in tubulin-motor interactions (e.g. gliding from β- towards α- tubulin or a rotation of motors around a vertical axis). We therefore moved the heads around each other by assuming the presence of three hinges, which we placed at positions Lys325, Ala339 and Lys354. Lys325 is at the C-terminal end of helix α6 and marks the beginning of the linker region, which appears to be rather flexible (Kull et al., 1996). Ala339 sits directly at the beginning of helix α7. Finally, Lys354 marks the beginning of the neck helix, which forms strong hydrophobic coiled-coil interactions, and which we assumed to maintain its coiled-coil conformation. Accordingly, we allowed the region between Ala339 and Lys354 to dissociate during processive movement. This partial melting of the coiled coil region (see Figure 8(d)) is essential because it allows moving one head over or around the other one without steric clashes. Since the neck and the gearbox region appear highly flexible (Kull et al., 1996; Rice et al., 1999) we used it as a kind of handle bar, which may flip back and forth during each step (Figure 8(d)), according to a model proposed earlier by Romberg et al. (1998).
During the progress of our own work even more compelling evidence for such a mechanism has now been demonstrated by tracking the position of gold-labels linked to the kinesin neck region of a monomeric motor head construct on helically averaged 3D maps of motor-microtubule complexes, similar to the ones shown here (Rice et al., 1999).

Conclusions

One of the direct consequences of our findings is that the helically averaged 3D maps shown here (and elsewhere) have to be interpreted with caution since they may be averages over different conformational states for the following reasons. (1) Assuming that both heads tend to bind to the microtubule surface, this is very unlikely to occur regularly in a helical fashion, otherwise we should observe 16 nm layer lines. (2) If the hand over hand model is correct we are likely to average over two or more different stepping patterns. Even if the loosely bound head should represent a truly microtubule-detached state there may be more than one of these states, which may occur randomly. (3) Every map of microtubules decorated with dimeric motor constructs (but not with monomers!) suffers to some extent from the fact that the nucleotide state of the two dimer heads cannot be regulated independently in a physiological way. Thus, conformational states which are in accordance with the principles of alternating head catalysis (Hackney, 1994; Gilbert et al., 1995, 1998; Hancock & Howard, 1999) can only be poorly simulated. Consequently, the appearance of loosely bound heads in 3D maps is difficult to interpret with respect to any physiologically relevant structural states. We were able to show, however, that the binding stoichiometry of kinesin motor domains to tubulin binding sites varies between 1.2 and 1.5 (see Table 1 and Figure 4) but never reaches a value of 2.0. This is clearly different to the situation found with ncd, where it is clear that each motor dimer occupies one tubulin binding site (=two heads per site). In the case of dimeric kinesins, however, we have to assume that the shapes of loosely bound motor domains revealed with helical reconstruction methods are the averaged result of different states and partial occupancy. Novel 3D reconstruction methods have to be applied in the future to investigate the true conformational states of dimeric kinesins complexed with microtubules.

STEM mass determinations and helical 3D reconstructions of microtubules decorated with dimeric kinesin constructs in the presence of AMP-PNP or no nucleotide (apyrase treatment) indicate a complex binding pattern in which both motor domains tend to bind to the microtubule surface along one protofilament. This conclusion is based on the following facts: (1) The pictures obtained with a crosslinked mutant kinesin shows a strong axial stabilization of individual protofilaments in microtubules, that still allows them to dissociate laterally (Figure 5). (2) Any kinesin dimer conformation, which reaches between two adjacent protofilaments would require a large-scale melting of the dimer neck regions to keep the two heads connected and would ask for consistently different conformational states from one head to the other. The overall dimer configuration would never even come close to the ADP-state configuration found in 3D crystals (Kozielski et al., 1997). (3) We have observed microtubule walls decorated with dimeric kinesin constructs which revealed a 16 nm periodicity, consistent with binding of two heads along one protofilament (Thormählen et al., 1998a; Hoenger et al., 1998). The consistent structural details in our maps between different nucleotide states argue against a possible head rotation on the microtubule surface, as well as, against a shift of binding sites from β- to α-tubulin. This indicates a steady overall binding conformation of the tightly bound heads during ATP hydrolysis with conformational changes taking place at the level of secondary structures. The microtubule binding pattern of dimeric kinesin is strikingly different from that one found with dimeric ncd (Sosa et al., 1997a; Hirose et al., 1998) and may constitute factors, which are related to the opposite directionality of these two molecular motors. Although little is known about the ncd walking mechanism it appears that the detailed molecular mechanisms, which move kinesin and ncd, have to be different from each other, not only with respect to the directionality, but probably also for the underlying molecular mechanism.

The model of kinesin motion currently favored is a walking process, which resembles a hand-over-hand mechanism (Cross, 1995; Block, 1998; Endow & Waligora, 1999). It does not completely exclude other possibilities but certainly makes them less likely. The walking model presented here illustrates the structural requirements and geometrical configurations at near-atomic scale, which have to be considered. The symmetry constraints from a homodimeric molecule such as kinesin require a complex coordination of the structural conformations within the two heads, which is not well understood. Consequently, any type of walking model has to include at least two substantially different ATP-driven molecular pathways, which allow for different structural conformations within the motor domains and their geometrical configurations to each other.

Materials and Methods

Expression of motor protein constructs

Plasmids coding for the rat kinesin constructs rK379 and rK354 were cloned and expressed as described in Kozielski et al. (1997). Briefly, to obtain pErK379 the BamHI-SauI-fragment of the rat kinesin gene (kindly provided by S.T. Brady, see Sack et al., 1997) was inserted...
into a derivative of the expression vector pET-3a (Studier et al., 1990) modified to contain the same sites with a stop codon. Plasmid pErK354 was cloned by PCR using plasmid pErK379 as a template. The construct were stop codon. Plasmid pErK354 was cloned by PCR using et al., 1990) modified to contain the same sites with a

Microtubule Binding Patterns of Dimeric Kinesins

Decoration of microtubules with kinesin

Tubulin was prepared by phosphocellulose chromatography preceded by a MAP-depleting step as described (Mandellkow & Mandellkow, 1985). Microtubules were polymerized for 20 minutes at 37°C in 80 mM Pipes (pH 6.8), 2 mM MgCl₂, at a concentration of 2.5 mg/ml and in the presence of 5% (v/v) DMSO, 2 mM GTP and 20 μM Taxol. Polymerized tubulin were washed twice by centrifugation in an Eppendorf centrifuge and resuspended in GTP free buffer. Decoration of polymerized microtubules with dimeric kinesin constructs was performed in solution at a final tubulin concentration of 0.5 mg/ml (~10 μM) and a final kinesin concentration of 0.8 mg/ml (~15-20 μM, depending on construct) and either in the presence of 2 mM AMP-PNP or after incubation with Apyrase. This stoichiometry was found previously (e.g. Hoenger & Milligan, 1997) to yield complete decoration. In order to check for an oversaturation effect on the appearance of loosely bound heads we were also decorating microtubules with dimeric Drosophila kinesin DHK392 at a ratio of 8 kinesin dimers per tubulin heterodimer (in contrast to 2:1 for our regular decoration experiments). Samples were incubated for two minutes and subsequently adsorbed to holey carbon grids for one minute and quick-frozen in liquid ethane by using a plunger essentially following the standard procedures described by Dubochet et al. (1985). Decoration of microtubules with rK379 and sK338 was performed directly on the grid to avoid bundling of tubules, either in the presence of 2 mM AMP-PNP, or in the absence of nucleotides (with apyrase (ten minutes/RT) in order to hydrolyze residual ATP. To this end microtubules at a concentration of 1 mg/ml were adsorbed on holey carbon grids for one minute, washed briefly, and incubated with a rK379 solution at 2 mg/ml to obtain the same stoichiometry as for rK354 = ca: 2 kinesin double heads per tubulin dimer) for two minutes and quick-frozen as described above.

ATPase Measurements with rK379-A339C

To characterize the A339C-mutant the microtubule-activated ATPase activity of both the reduced and the oxidized form were tested and compared to that of the wild-type construct.

In order to limit dissociation of the kinesin neck-helix measurements were performed at 0.2 μM rK379 dimer (both wild-type and mutant). Microtubules were diluted to 0.125-7.5 μM from a 150 μM stock previously polymerized for 20 minutes at 37°C in the presence of 1 mM GTP and 20 μM Taxol. ATP hydrolysis was initiated by adding a mixture of kinesin and Mg-ATP in buffer (final concentrations 0.2 μM kinesin dimer, 1 mM Mg-ATP, 5 mM MgCl₂, 50 mM NaCl, 20 mM Pipes (pH 6.9), 20 μM Taxol). After one minute the reaction was quenched by adding an equal volume of 0.6 M perchloric acid. An aliquot was used to determine the released phosphate by the malachite green assay as described by Kodama et al. (1986).

Cryo-electron microscopy

Cryo-electron microscopy was performed on a Philips CM120-BioTWIN microscope, using a Gatan-626 cryo-holder. Images were recorded on Kodak SO-163 electron microscopy film at a defocus around ~1.5-2.0 μm. On the CM120-BioTWIN microscope this defocus range places the first zero node of the contrast transfer function between 1/27 Å and 1/20 Å.

Image processing and 3-D reconstruction

Due to the variations in lattices among microtubules (see Mandellkow et al., 1986; Wade et al., 1990; Sosa & Milligan, 1996; Sosa et al., 1997b) we screened images for microtubules which exhibited a Moiré pattern (see Mandellkow & Mandellkow, 1985; Wade et al., 1990) that corresponds to a 15 protofilament/2-start helical microtubule (Figure 2(a); see also Sosa et al., 1997b). These microtubules accounted for approximately 5% of the total number of microtubules present in a solution when polymerized in the presence of 5% DMSO. 15-protofila ment/2-start helical microtubules also exhibit a characteristic diffraction patterns as shown in Figure 2(b). Micrographs were digitized using a Zeiss-SCAI scanner operated at a pixel size of 21 μm on the negative, which corresponds to 0.5676 nm on the sample. Suitable 15-protofilament/2-start microtubules were helically reconstructed by using the program suite PHOELIX (Whittaker et al., 1995). Some of them were long enough to be pre-screened for multiple seams in the microtubule wall by back projection (Sosa & Milligan, 1996), but such cases were not observed. One dataset constitutes either a near or a far side of an intact microtubule which is already averaged over four to eight repeats. The microtubules used here had an average length of approximately 1.2 μm. Accordingly our maps are averages of between 17,000 and 30,000 asymmetric units (5β-tubulin-motor complexes). All datasets were truncated to a maximum resolution of 25 Å. 3D maps were visualized by using SUPRIM (Schroeter and Bretaudiere, 1996) and VolVis (SUNY Stonybrook). High-resolution structures were imaged using MoSscript (Kraulis, 1991). The polarity of the maps presented here was determined according to Hoenger & Milligan (1996). All maps are shown with their plus-end up.
STEM mass determinations

STEM mass determinations were performed on a Vacuum Generator-HB5 scanning transmission electron microscope operated at an acceleration voltage of 80 kV. The preparation was carried out as described above for cryo-EM. Samples were adsorbed to glow-discharged microscope operated at an acceleration voltage of 80 kV. Vacuum Generator-HB5 scanning transmission electron microtubule binding patterns of dimeric kinesins

References


*Edited by W. Baumeister*

(Received 10 December 1999; received in revised form 10 February 2000; accepted 13 February 2000)