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Structural comparison of dimeric Eg5, *Neurospora* kinesin (Nkin) and Ncd head–Nkin neck chimera with conventional kinesin

Keiko Hirose, Ulrike Henningsen[1], Manfred Schliwa[1], Chikashi Toyoshima[2], Takashi Shimizu[3], Maria Alonso[4], Robert A.Cross[4] and Linda A.Amos[5,6]

National Institute of Advanced Interdisciplinary Research, Tsukuba 305-8562, [2]Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113, [3]National Institute of Bioscience and Human Technology, Tsukuba 305, Japan, [1]Adolf-Butenandt-Institüt Zellbiologie, Ludwig-Maximilians-Universität, München, Germany, [4]Marie Curie Research Institute, Oxted, Surrey and [5]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

[6]Corresponding author
e-mail: laa@mrc-lmb.cam.ac.uk

Cryo-electron microscopy and 3D image reconstruction of microtubules saturated with kinesin dimers has shown one head bound to tubulin, the other free. The free head of rat kinesin sits on the top right of the bound head (with the microtubule oriented plus-end upwards) in the presence of 5′-adenylylimido-diphosphate (AMPPNP) and on the top left in nucleotide-free solutions. To understand the relevance of this movement, we investigated other dimeric plus-end-directed motors: *Neurospora* kinesin (Nkin); Eg5, a slow non-processive kinesin; and a chimera of Ncd heads attached to Nkin necks. In the AMPPNP (ATP-like) state, all dimers have the free head to the top right. In the absence of nucleotide, the free head of an Nkin dimer appears to occupy alternative positions to either side of the bound head. Despite having the Nkin neck, the free head of the chimera was only seen to the top right of the bound head. Eg5 also has the free head mostly to the top right. We suggest that processive movement may require kinesins to move their heads in alternative ways.

Keywords: cryo-electron microscopy/Eg5/kinesin/microtubules/Ncd

Introduction

The kinesin family of motor proteins includes members that move in opposite directions along microtubules (MTs), in spite of possessing catalytic domains of highly homologous sequence and atomic structure (Kull *et al*., 1996; Sablin *et al*., 1996). Motors that move towards the plus end of MTs, such as conventional kinesin, have their catalytic domains (heads) connected to a stalk domain via a 'neck' segment at their C-termini, while reverse motors, such as Ncd, have the stalk domain and neck at the N-termini of their catalytic domains (for review see Endow, 1999). A striking feature of conventional kinesin is its processivity, that is, the ability of the dimeric motor to move along an MT, for 100 ATPase cycles or more, without detaching at any point in the cycle. It is thought to do this by a hand-over-hand means of progression. In contrast, most other members of the family, such as Ncd (encoded by *non claret disjunctional*) and Eg5, a slow plus-end-directed motor, make a single or very few steps before detaching (Case *et al*., 1997; Crevel *et al*., 1997).

The necks of kinesin and Ncd differ in the atomic structures of these proteins: crystallized with ADP bound, the kinesin neck consists of a β-stranded region (the 'neck linker') that associates with the catalytic core and the 'neck coiled coil' (Kozielski *et al*., 1997; Sack *et al*., 1997); in contrast, the Ncd neck seen in the dimer structure forms a coiled coil sandwiched between two catalytic domains (Sablin *et al*., 1998; Kozielski *et al*., 1999). However, although kinesin's neck is attached to the C-terminus of the catalytic domain and Ncd's neck to the N-terminus, both interact with roughly similar regions of the catalytic core surface (Sablin *et al*., 1998).

The construction of various mutant and chimeric motors has shown that both the position and the sequence of the neck domain are important in determining the direction of movement. Dimeric chimeras consisting of Ncd catalytic domains connected at their C-termini to the neck-linker and stalk domains from a kinesin, either *Neurospora* kinesin (Nkin; Henningsen and Schliwa, 1997) or human conventional kinesin (Case *et al*., 1997), move towards the plus end of an MT. A complementary chimera, consisting of kinesin catalytic domains connected at their N-termini to Ncd necks, moved towards the minus end except when a few crucial residues were mutated, when movement towards the plus end was restored (Endow and Waligora, 1998). It was also shown that a dimeric Ncd construct with the normal neck replaced by a random chain moved towards the plus end, although very slowly (Sablin *et al*., 1998). These studies showed (i) that the catalytic domains of kinesin and Ncd both have an intrinsic tendency to move towards the plus end of MTs, although the movement was extremely slow without a proper neck, and (ii) that the Ncd neck sequence is crucial in reversing the direction of movement.

In the atomic structures of dimeric constructs, the heads of kinesin and Ncd interact differently. An extensive array of residues on each head of dimeric Ncd (Sablin *et al*., 1998; Kozielski *et al*., 1999) contacts either side of the coiled-coil neck but the heads make no direct contact with each other. The two heads of dimeric Ncd molecules on MTs also appear to be connected indirectly through the neck domain (Hirose *et al*., 1998, 1999a). In the crystal structure of dimeric kinesin (Kozielski *et al*., 1997), two identical heads are related by an ~120° rotation and the end of each neck linker attaches at a different angle to the coiled-coil domain. The heads also contact one another directly in a non-equivalent relationship. The interactions must be quite weak since both heads and necks appear to

move when dimeric kinesin is free in solution (Marx *et al*., 1998; Rice *et al*., 1999; Stone *et al*., 1999). When bound to MTs, if the interactions are stabilized during freezing for electron microscopy (EM), kinesin dimers appear to be arranged in much the same way as in the crystal structure (Hirose *et al*., 1999a).

Several lines of evidence point to the importance of communication between the heads in the kinesin stepping mechanism. Transient kinetic measurements indicate that ATP binding to one head of kinesin (Hancock and Howard, 1999) or the hydrolysis of ATP by Nkin (Crevel *et al*., 1999) accelerates ADP release from the other head. 5′-adenylylimido-diphosphate (AMPPNP), a non-hydrolysable analogue of ATP, also accelerates ADP release, albeit to different extents in rat and human kinesin and Nkin. These effects have not been reported for motors such as Ncd and Eg5 (Case *et al*., 1997; Crevel *et al*., 1997). Structural studies using cryo-EM on kinesin or Ncd complexed with MTs under different conditions have shown a major left-to-right rearrangement of the two heads in the case of kinesin (Hirose *et al*., 1999a) but not in the case of Ncd (Hirose *et al*., 1998). Since any structural difference between kinesin and Ncd may be related to directionality or to processivity, we have now studied some other dimeric constructs: Nkin, Eg5, a slow kinesin homologue and an Ncd–Nkin chimera. Both of the latter move towards the plus end of the MT with little or no processivity (Crevel *et al*., 1997; Henningsen and Schliwa, 1997). Our data provide new insight into the basis of processivity, as well as into direction reversal in the Ncd–Nkin chimera.

Results

Electron micrographs of MTs incubated with Nkin, the Ncd–Nkin chimera (NNΔ448; Figure 1A) or with the Eg5–glutathione *S*-transferase (GST) construct (EgΔ437–GST) showed regularly decorated MTs, similar to those obtained with other dimeric constructs (Hirose *et al*., 1996, 1998, 1999a). The specimens had been rapidly frozen in the presence either of apyrase, to produce the nucleotide-free state, or of 2 mM AMPPNP, to produce an ATP-like state. The fusion protein was used in the case of EgΔ437–GST because of solubility problems with Eg5 expressed alone. Images of MTs decorated with the fusion protein seemed to show some extra density at higher radii but since it is not well ordered, a clear contribution from the GST in the reconstructed images was not expected. Diffraction patterns from all images showed the usual 8 nm longitudinal periodicity of decorated MTs. Amplitude and phase distributions along the layer lines in calculated Fourier transforms were also similar to those obtained previously from MTs decorated with rat kinesin constructs, KΔ401 and KΔ430 (Hirose *et al*., 1999a) or Ncd, NΔ295–700 (Hirose *et al*., 1998). The differences between all these types of data are subtle but they give reproducible differences in structure at high radius in 3D density maps.

Figure 2A shows that dimeric Nkin in the AMPPNP state is very similar to rat kinesin in this state (Figure 2E), with one head (H1) directly bound and the other (H2) tethered to the top right of H1, in our standard orientation, with the plus end of the MT towards the top. In an average of 24 individual maps of the apyrase state (not shown but very similar to Figure 2D), density representing the tethered head of Nkin appeared to be tenuously attached to all three of the closest directly bound heads. However, in many of the individual maps, the tethered head appeared more strongly connected to the top left or top right of a directly bound head. In none of the individual maps did the

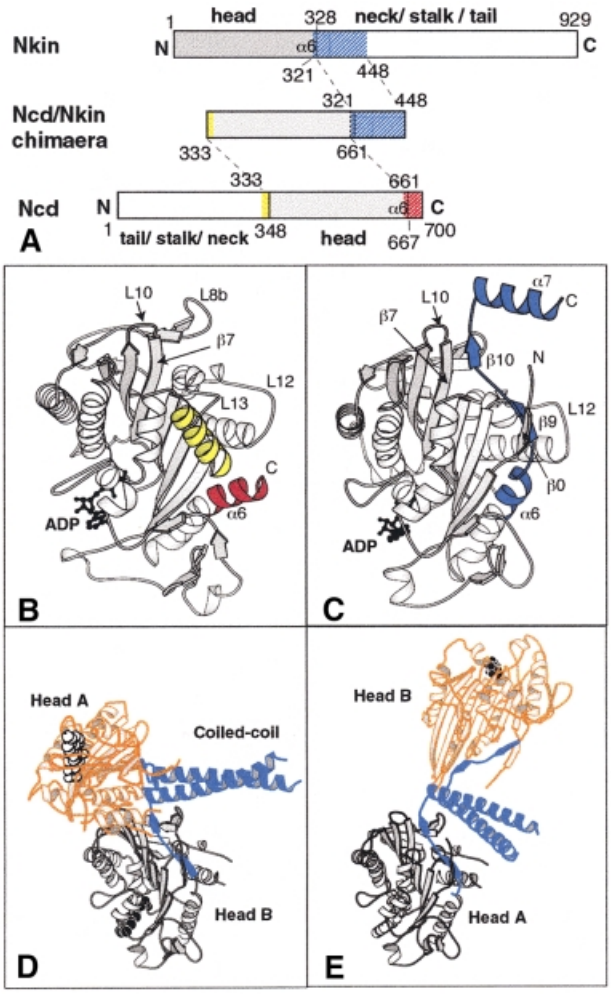


Fig. 1. (**A**) Construction of the Ncd–Nkin chimera (NNΔ448), consisting of residues 333–661 of Ncd (light grey) followed by residues 322–448 of Nkin (coloured in blue), a fast kinesin molecule (Henningsen and Schliwa, 1997) (see also Table I). The construct is dimerized through the Nkin coiled-coil stalk region. It also includes a part (residues 333–348; coloured in yellow) of the Ncd neck [residues 327–348 (Endow and Waligora, 1998)] but not enough to dimerize the motor domains at this end of the chain. The C-terminal residues, including part of helix α6, which were removed from Ncd, are coloured red. (**B** and **C**) Ribbon diagrams of the atomic structures of Ncd (residues ~333–672) (Sablin *et al*., 1998) and rat kinesin (residues 2–351) (Sack *et al*., 1997); Nkin is assumed to be like kinesin. The catalytic domains (uncoloured) are shown with associated neck linkers. The Ncd neck linker (coloured yellow) is α-helical in this crystal structure but was disordered in the Ncd monomer crystals (Sablin *et al*., 1996). The chimeric motor consists of the Ncd catalytic domain, joined in the middle of helix α6 (with the red segment removed) to the neck and part of the coiled-coil tail of Nkin (coloured blue). Note that the neck of Ncd replaces the N-terminal strand β0, which associates with β9 of the kinesin neck. (**D** and **E**) Two views of the atomic structure of the kinesin dimer (Kozielski *et al*., 1998). Neck domains are coloured blue. Exchanging the relative relationship of heads A and B causes a large shift in the position of the upper head but only a small conformational change in each neck linker, close to its connection to the coiled coil.

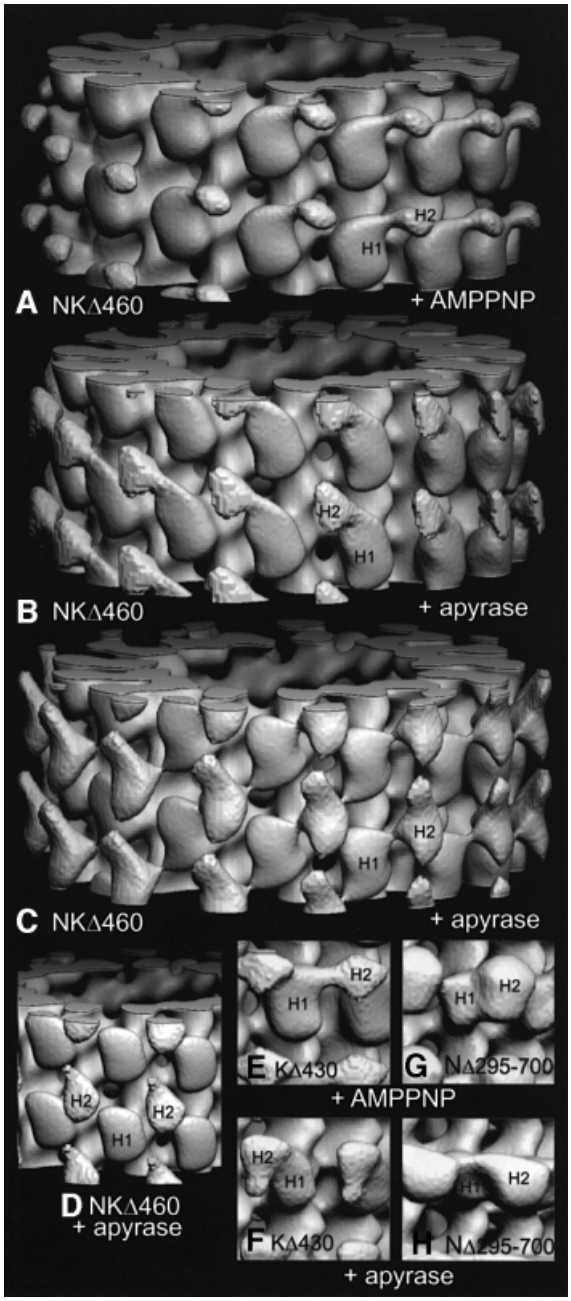


Fig. 2. Surface representations of reconstructed images of MTs decorated with dimeric motors. In all cases, one of the two heads (H1) is attached directly to the MT, while the other (H2) is tethered to the first head. (**A**) *Neurospora* kinesin (NKΔ460) in the presence of AMPPNP. (**B**–**D**) Three arrangements seen for Nkin in the absence of nucleotides, produced by averaging different groups of individual maps. The tethered head, H2, may appear to be attached to H1 on its right or left, or be suspended between two bound heads. For comparison, dimeric rat kinesin (KΔ430) (**E** and **F**) and Ncd (NΔ295–700) (**G** and **H**) are shown in the same two nucleotide states (from Hirose *et al*., 1998, 1999a).

tethered head appear to be attached specifically to the bottom of a bound head. Three separate averages were therefore made of maps showing a top-right or top-left connection and others where the connection was ambiguous. The results are shown in Figure 2B–D. The average of the third group (Figure 2D) resembled the overall average. It is likely that all the images included tethered heads in the top-right and top-left positions but that one or other state predominated in many cases. The top-left configuration (Figure 2B) appears to be similar, although not identical, to that of conventional kinesin in the empty state (Figure 2F; Hirose *et al*., 1999a). The top-right configuration (Figure 2C) has not been seen before; the tethered head (H2) is tilted at a different angle from the AMPPNP state of kinesin or Nkin and its size appears larger (less disordered), closer to that of the directly bound head (H1).

Figure 3A–D shows the surface features of the Ncd–Nkin chimera and Eg5 in two nucleotide states. In both cases, the position of the tethered heads in the presence of AMPPNP (Figure 3A and C) is similar to that seen for kinesin (Figure 2E) and Nkin (Figure 2A), while in the absence of nucleotide (Figure 3B and D) it is different from that of kinesin (Figure 2F) but quite similar to the alternative position in Nkin (Figure 2C). The map showing EgΔ437–GST in the absence of nucleotide (Figure 3D) has an extra density peak whose origin is unclear. It may represent part of the neck–tail–GST extension or be a minor position for the tethered head.

A structural change between different nucleotide states was clear with kinesin and Nkin, but it is more subtle with Eg5 or Ncd–Nkin, where the main effect of removing or adding nucleotide is a change in the position and angle of the tethered head. In the case of Ncd–Nkin, for example, it is roughly vertical in the absence of nucleotide (Figure 3B) but moves up and rotates clockwise by ~40° in the presence of AMPPNP (Figure 3A). Small changes observed in the actual shapes of the heads may not be significant but there is clearly an overall movement.

In AMPPNP maps of conventional kinesin (Hirose *et al*., 1996; Figure 2E) or Nkin (Figure 2A), the density of the tethered head is weak compared with that of the bound head, probably because the tethered heads are disordered. In contrast, the tethered heads of Ncd–Nkin (Figure 3A) and Eg5 (Figure 3C) appear to be in a relatively fixed position. The tethered head of the chimera appears to make contact with the bound head of another dimer, which is attached to the next protofilament. Because Eg5 is tilted forwards by a few degrees, it does not make this contact.

In addition to the Nkin neck, the chimeric motor construct (Figure 1) includes the Ncd neck that, in the dimeric Ncd crystal structure, forms a coiled coil continuous with the stalk domain; the catalytic domains in the crystal are apparently held in position on either side of the coiled coil by interactions along its length. Since the relative position of the two heads on MTs resembles that seen for kinesin, the kinesin neck at the C-terminus must be responsible for dimerization and it is unlikely that the Ncd neck residues interact with each other in this construct.

Discussion

The present work shows MTs decorated with dimers of Nkin, Eg5 or the Ncd head–Nkin neck chimera, each in two nucleotide states. All show tethered head density attached to the top of a directly bound head. Figure 4 summarizes our results for the dimeric motors we have studied, including previous work on Ncd and rat kinesin (Hirose *et al*., 1998, 1999a).

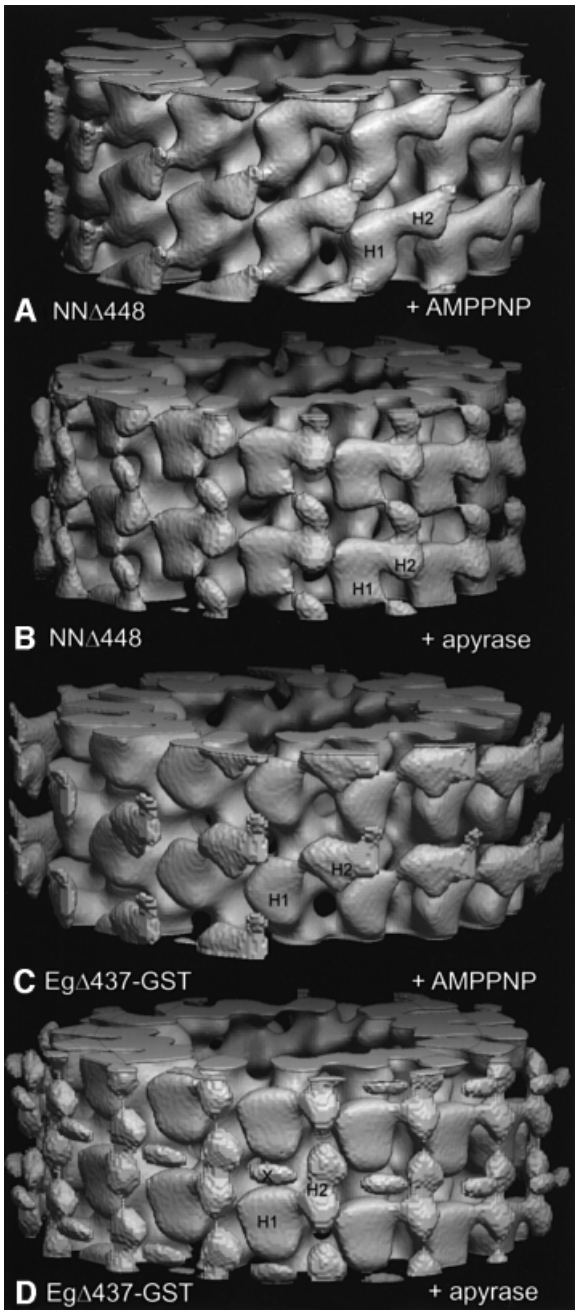


Fig. 3. Surface representations of reconstructed images of MTs decorated with non-processive, plus-end-directed dimeric motor domains. (**A** and **B**) The Ncd–Nkin chimera (NNΔ448) in the presence of AMPPNP and in the absence of nucleotides. (**C** and **D**) Eg5–GST (EgΔ437–GST) in these two states. As in Figure 2, head H1 is attached directly to the MT, while H2 is tethered to H1.

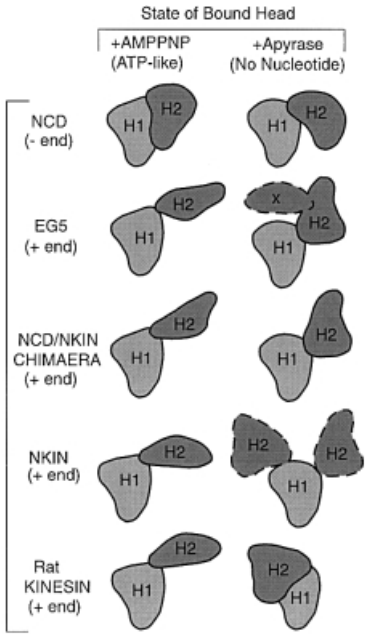


Fig. 4. Schematic comparison of the structural changes observed between different nucleotide states for various dimeric motors attached to MTs. The results for Nkin and the non-processive dimers of Eg5 or the chimera, consisting of the Ncd catalytic domain attached to the Nkin neck, are from the present work. Changes in the normal Ncd dimer are summarized from Hirose *et al.* (1996, 1998); those in rat kinesin are from Hirose *et al*. (1996, 1999a).

Comparison of dimeric motors in the ATP-like state

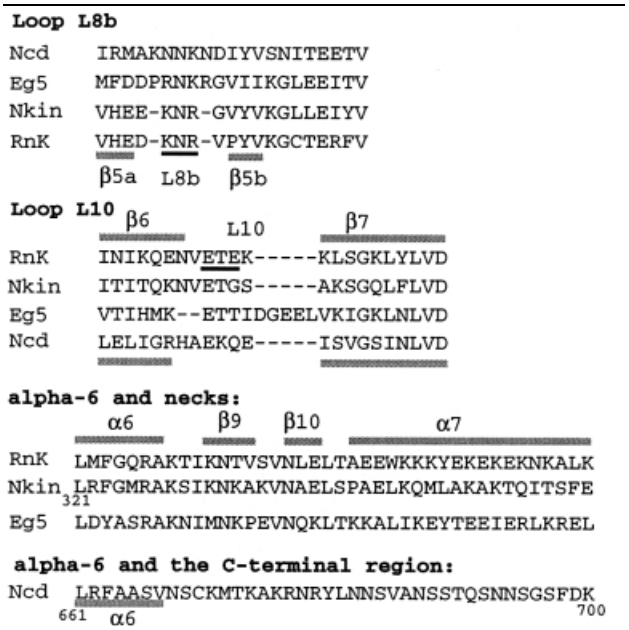
For all plus-end-directed dimeric motors, the tethered head is positioned to the top right of a bound head containing AMPPNP. This position for the tethered head is apparently dictated by the neck linker of the first head, according to the results of Rice *et al*. (1999), who labelled the neck linker of a monomeric kinesin in order to track its movement; cryo-EM images of the ATP state showed that gold label attached to the β10 region of the neck was on the top right of a head, very close to where the second head emanates in our maps. EPR labelling of the same site (Rice *et al*., 1999) indicated that the neck linker was firmly bound only when a motor in the ATP state interacted with MTs. Our images suggest that Nkin and Eg5 neck linkers also make this firm attachment, even when the Nkin neck is associated with the 'wrong' catalytic domain.

In the crystal structure of dimeric kinesin with bound ADP (Figure 1D and E), β9/β10 in the kinesin neck linker associates with parts of the catalytic core including β0 and β7 (Kozielski *et al*., 1997). Ncd's catalytic domain lacks β0 and has some different residues in the L10 loop and start of the β7 strand (Table I). However, when the catalytic domain changes its conformation through binding to tubulin and ATP, the neck linker may interact strongly with more highly conserved residues, or part of it might even interact with part of the MT surface such as the projecting C-termini of the tubulin monomers (Nogales *et al*., 1999). The tethered head always appears smaller than the bound head in the ATP-like state and must be fairly mobile. Although the neck linker of the bound kinesin may be firmly fixed, the tethered head, containing ADP, is only weakly attached to its own neck linker.

Comparison of dimeric motors in the empty state

In both examples of processive motors, conventional kinesin and Nkin, the tethered head can take up positions on the top left of the bound head in the nucleotide-free state. None of the non-processive motors showed this arrangement. The tethered head of Nkin was also seen occupying a top-right position in the nucleotide-free state, with a roughly equal probability to being in the top-left position. Thus, although we have seen the tethered head of

Table I. Sequence comparison of rat kinesin (RnK), *Neurospora* kinesin (Nkin), Eg5 and Ncd in three regions



The upper two sequences include loops L8b and L10, which appear to interact with each other in the crystal structure of dimeric kinesin (Kozielski *et al*., 1997). The bottom comparison shows part of α6 and the neck in kinesins and Eg5, and the corresponding region in Ncd. The Ncd–Nkin chimera studied here consists of the catalytic core of Ncd (up to residue 661) connected to the neck of Nkin (from residue 321).

conventional kinesin in only one position relative to a bound head in the empty state, it is likely that it will, under different circumstances, have probabilities of lying to either side. Rice *et al*. (1999) identified a third position for their neck-linker label, at the bottom right of the bound head. We saw no evidence that the tethered heads of either kinesin or Nkin can occupy this position, but it may occur under suitable conditions.

The tethered head of the Ncd–Nkin chimera in the empty state seems to be firmly fixed to the top right of the directly bound head. That of Eg5 appears less firmly fixed and the density labelled 'X' in Figure 3 may indicate a second position near the top centre. However, neither non-processive motor showed tethered-head density to the top left of the bound head. As discussed later, a movement from side to side of the tethered head (and, by implication, the neck of the bound head) does not seem to be essential for progress towards either end but is probably associated with processive movement.

Directionality

The results obtained from Eg5 and from the chimera (Figure 3) confirm that the sequence and position of the neck have a dominant effect on the arrangement of the two heads. The tethered head of a plus-end-directed dimer is always seen closer to the MT plus end, in an arrangement similar to that of kinesin bound to an MT in the presence of AMPPNP. Its proximity to the plus end is in contrast to the Ncd tethered head, which points toward the minus end (Figure 2G and H). It has been suggested previously (Hirose *et al*., 1996) that the position of the tethered head might provide a structural bias that guides the tethered head to the next tubulin site in the right direction. Case *et al*. (1997) estimated that the run lengths of Ncd and their Ncd–kinesin chimera are unlikely to be more than six tubulin subunits. However, if they can move continuously even for just a few steps, having the unbound head in the direction of movement would help directed motility.

It is proposed that the neck regions change conformation in response to changes in the catalytic core during the MT-activated ATPase cycle (Sablin *et al*., 1998; Rice *et al*., 1999). Studies using mutated kinesin constructs suggest that dimeric kinesin cannot step forwards along an MT unless the neck of the bound head is in the top right position in the ATP/ADP·P_i state (Rice *et al*., 1999). Thus, it seems important for a plus-end motor to have the neck (and tethered head) in this position when the bound head is in the presence of AMPPNP (thought to mimic either ATP or ADP·P_i). In AMPPNP, all of the plus-end-directed constructs we have studied do indeed have their tethered heads in a similar position to that of kinesin.

Comparison of the structures in the two nucleotide states shows some common structural changes for both plus-end and minus-end motors. In fact, the directly bound heads of all the motors we have studied so far appear to undergo similar nucleotide-associated changes. When AMPPNP is bound to the attached head, the point connecting the two heads always moves up a little towards the MT's plus end compared with the empty state position (Figure 4). The ways in which kinesin or Ncd in different nucleotide states interact with tubulin are also homologous (Hirose *et al*., 1999b). The differences we have seen between different motors simply occur in the angles at which the tethered heads are attached.

Processive and non-processive movement

Dimers of the chimeric motor do not process, in spite of having the neck of a processive motor and having tethered heads in a suitable position in the AMPPNP state. Our results suggest that having an Nkin neck is not enough to produce all the kinesin-like or Nkin-like arrangements of the two heads. In the nucleotide-free states, the tethered heads of Eg5 and Ncd–Nkin never appear to shift over to the left (Figure 4). The images in Figure 3 suggest a possible direct interaction between the heads that may restrict their movement and that of the neck linker. Eg5 may also be entirely non-processive, even though its neck linker has a significant sequence similarity to those of kinesin and Nkin (Table I). As mentioned in the Introduction, processivity also requires a close kinetic coordination between the two heads, which may only be true for kinesin and Nkin.

In the empty state, only the tethered heads of kinesin and Nkin appear to the left of the attached heads, while the tethered heads of other motors stay towards the right side of the attached heads. It thus seems unlikely that a large left–right movement is a necessary feature of the cycle of ATP hydrolysis. Indeed, the results for Nkin indicate that a large movement of the tethered head may occur without any significant conformational change in the catalytic domain of the attached head. One possibility is that the left–right movement of kinesin's or Nkin's tethered head

reflects a swapping of the relative relationship between 'head A' and 'head B' of the kinesin dimer crystal structure (Figure 1D and E). It seems very likely that the heads of a kinesin dimer bound to an MT have a similar asymmetrical relationship. For example, different maps of MTs decorated with dimeric kinesin in the ADP-filled state (Arnal and Wade, 1998; Hirose *et al*., 1999a) show alternative arrangements that can be related to the crystal structure.

The best EM map, to date, of MTs decorated with kinesin is that of Kikkawa *et al*. (2000) showing the monomer of mouse conventional kinesin in the ATP-like state at 22 Å resolution. The map has a protruding feature that points up and outwards from the top of the motor domain, at a somewhat different angle from an equivalent feature in the accompanying KIF1A motor domain structure. The feature corresponds well with the position of loop L10 according to our docking of the kinesin dimer (see Figure 1D, which represents the front view). In our map of dimeric kinesin bound to MTs in the presence of ADP (Hirose *et al*., 1999a), 'head B' fitted into the directly bound motor domain while 'head A' was in the same position as the tethered head. If, instead, 'head A' were docked into the directly bound head, a tethered 'head B' would be positioned on the right (Figure 1E) and would resemble maps showing dimeric kinesin in the presence of ADP and free phosphate ions (Arnal and Wade, 1998; K.Hirose and L.Amos, unpublished result). With the attached head docked in this orientation, there is no steric conflict between the MT and the tethered head in either of its alternative positions. The atomic structure is only known for dimeric kinesin with ADP bound but a similar asymmetry between the two heads may persist throughout the hydrolysis cycle. Thus, kinesin dimers in other nucleotide states may have alternative conformations, with probabilities that vary according to slight environmental differences.

We do not understand exactly how kinesin-like motors advance along an MT, except that the neck domains are important for both the direction of movement and for processivity (Rice *et al*., 1999). However, it is clear that the movement of one head past another must introduce a twist in the linkage between them. A dimer whose tethered head is only able to park in one position might always move in the same way and be forced to detach after a fairly small number of cycles. To avoid a build-up of tension, alternate steps need to reverse the twist introduced in the previous step. A possible way to accomplish this is illustrated in Figure 5. The 3D images of Eg5, Ncd and the Ncd–Nkin chimera suggest that they may lack processivity because of an inability of the heads to interact with one another in alternative ways.

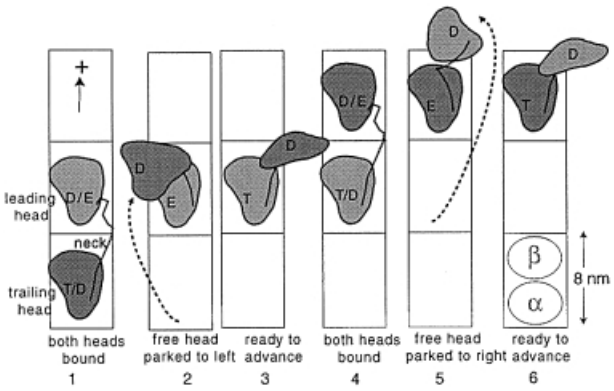


Fig. 5. Tentative scheme for avoiding excessive twisting of the neck domain during processive movement of dimeric kinesin. ATP (T) binds to the head attached to an MT (3 or 6), causing its neck to extend forwards and thus guiding the other head into the lead position (1 or 4). After hydrolysis of ATP, the trailing head detaches and, now containing only ADP (D), swings past the point where the two necks join and 'parks' briefly on the bound head (2 or 5) before moving ahead to interact with the next α/β tubulin dimer. Having lost its ADP there, the empty nucleotide-binding site (E) is ready to receive a new ATP molecule. The direction in which the moving head swings and whether its parked position resembles Figure 1D or E may be different for alternate steps (2 versus 5).

Materials and methods

The Nkin construct, NKΔ460, was purified as described by Crevel *et al*. (1999). The chimera consisting of the Ncd catalytic domain fused in the middle of helix α6 with the neck domain plus the coiled-coil stalk of Nkin (Henningsen and Schliwa, 1997) was truncated at Nkin residue 448 to produce a shorter dimeric construct (NNΔ448) as shown in Figure 1A. The expressed protein was purified on an SP-Sepharose column and then a Q-Sepharose column. NNΔ448 was retained by both columns at low ionic strength and was released at 0.15–0.2 M NaCl, which made the purification procedure very efficient. The EgΔ437–GST construct consisted of residues 12–437 of Eg5 fused at the C-terminus with GST. The protein was expressed and purified as described (Lockhart and Cross, 1996). The kinesin construct KΔ430 was that used by Hirose *et al*. (1999a).

MTs were assembled from purified pig brain tubulin in a polymerizing solution [80 mM PIPES pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM GTP, 5% dimethylsulfoxide (DMSO)], stabilized with 20 μM taxol, centrifuged and resuspended in a solution without GTP. DMSO was added in order to increase the proportion of 15-protofilament MTs (Ray *et al*., 1993). MTs diluted in MES solution (60 mM MES, 5 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT, 10 μM taxol pH 6.5) were applied to an EM grid coated with a holey carbon film, and NKΔ460, NNΔ448 or EgΔ430 was added to give a final concentration of 10 μM. For freezing in the no-nucleotide state, the MT–motor mixture was incubated on the grids with 2 U/ml apyrase and then rapidly frozen by plunging the grids into an ethane slush. For the AMPPNP state, the MT–motor mixture was incubated on the grids with 2 mM AMPPNP prior to freezing.

The grids were examined using a Gatan or Oxford cold stage in a Philips EM 420 electron microscope operating at 120 kV or a Hitachi HF-2000 electron microscope operating at 200 kV. Images were photographed at a magnification of 36 000 or 40 000× and then scanned in 28 μm steps. Images of MTs with 15 protofilaments were selected and processed as described (Hirose *et al*., 1998).

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