Coupled chemical and mechanical reaction steps in a processive *Neurospora* kinesin

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We show using single molecule optical trapping and transient kinetics that the unusually fast *Neurospora* kinesin is mechanically processive, and we investigate the coupling between ATP turnover and the mechanical actions of the motor. Beads carrying single two-headed *Neurospora* kinesin molecules move in discrete 8 nm steps, and stall at ~5 pN of retroactive force. Using microtubule-activated release of the fluorescent analogue 2-(3')-O-(N-methylanthramiloyl) adenosine 5'-diphosphate (mantADP) to report microtubule binding, we found that initially only one of the two motor heads binds, and that the binding of the other requires a nucleotide ‘chase’. mantADP was released from the second head at 4 s\(^{-1}\) by an ADP chase, 5 s\(^{-1}\) by 5'-adenylylimidodiphosphate (AMPPNP), 27 s\(^{-1}\) by ATP\(_{\gamma}\)S and 60 s\(^{-1}\) by ATP. We infer a coordination mechanism for molecular walking, in which ATP hydrolysis on the trailing head accelerates leading head binding at least 15-fold, and leading head binding then accelerates trailing head unbinding at least 6-fold.

**Keywords**: kinesin/microtubules/molecular motor/optical trapping/processivity

Introduction

Single molecules of ubiquitous kinesin (ukinesin; uKHC, KIF5b) move long distances (upwards of a micron) along microtubules (MTs) without detaching (Howard et al., 1989; Vale et al., 1996; Visscher et al., 1999), because of a property termed mechanical processivity. Current data suggest that ukinesin may ‘walk’ along MTs, touching down with alternate head domains to binding sites spaced 8 nm apart along the MT protofilament axis. The 8 nm step distance has been measured directly by optical trapping nanometry (Svoboda et al., 1993, Kojima et al., 1997) and corresponds to the spacing of tubulin heterodimers along the MT protofilament axis. The molecular mechanism of force generation by kinesins is not yet understood, although conformational changes corresponding to the biochemical kinetic events of ATP turnover are beginning to be characterized (Hirose et al., 1995, 1998; Arnal et al., 1996; Alonso et al., 1998). Similarly, the detailed mechanism by which the mechanical action of walking ukinesin is coordinated is not clear, although recent data have indicated that ATP binding to the trailing (rear) head of the molecule is required for leading (front) head attachment (Hackney, 1994; Ma and Taylor, 1997; Gilbert et al., 1998).

Several other members of the kinesin motor superfamily do not appear to exhibit ukinesin-like mechanical processivity (Crevel et al., 1997; Stewart et al., 1998). A recent report described processivity from a single-headed kinesin family motor called KIF1A (Okada and Hirokawa, 1999), but the character of the motion in this case was different, being a biased one-dimensional diffusion that would presumably lose ground under load. Here we use the term ‘mechanical processivity’ to mean movement via a repetitive mechanical stepping action that is resistant to retroactive loads.

*Neurospora* kinesin (Nkin) has an unusually long Pro,Gly-rich neck region insert, a structural feature that may correlate to mechanical processivity (Crevel et al., 1997). Nkin is of additional interest in that it is amongst the fastest kinesins so far described (Steinberg and Schliwa, 1995), and it seemed reasonable to expect that its speed might require it to have a particularly effective head–head coordination mechanism. We recorded mechanical transients from single Nkin molecules using optical trapping nanometry on a custom-built microscope, and found that Nkin is indeed mechanically processive. We went on to probe the mechanism of coordination of the leading and trailing heads using the MT-activated release of the fluorescent analogue mantADP as a reporter signal for MT binding. We found, as previously reported for ukinesin (Hackney, 1994; Ma and Taylor, 1997; Gilbert et al., 1998), that MT-activated release of mantADP from the leading head of Nkin requires ATP binding to the trailing head. In contrast to current ukinesin models, we found that for Nkin the release of leading head ADP ordinarily requires hydrolysis of trailing head ATP. The requirement for hydrolysis sets the Nkin mechanism apart from that proposed for ukinesin, but, as we discuss, it is possible that both sets of findings can be subsumed into a single scheme in which the reactions leading to ADP trapping progressively increase the probability of trailing head detachment.

Results

**Solution molecular weights of expressed proteins**

Nkin constructs of various lengths were prepared, both in fusion with glutathione S-transferase (GST) and without. The solution association state of the expressed and purified proteins was checked using glycerol gradient centrifugation and analytical gel filtration on Superose 12. Both techniques confirmed that Nkin360 behaves like a monomer in solution and that Nkin460GST is dimeric (Table I).
Steady-state binding of Nkin to MTs

The binding stoichiometries and dissociation constants for MTs of the constructs were measured using a MT pelleting assay (Figure 1). Data are summarized in Table II. The dissociation constants for Nkin binding to MTs in different nucleotides showed the same pattern as observed previously for other kinesin family motors (Shimizu et al., 1995; Crevel et al., 1996; Rosenfeld et al., 1996), with ADP being the weakest binding state. All of the Nkin dissociation constants were ~10-fold tighter than for other kinesins, perhaps indicating a slow dissociation rate constant compared with ukinisin•ADP (see kinetic arguments below). The binding in 5’-adenylylimidodiphosphate (AMPPNP) is so tight that it was difficult to get an accurate value using the pelleting assay, and the values are therefore given as a limit. The data are consistent with previously proposed kinetic schemes in which motor alone and motor•ATP are strongly bound states, and motor•ADP is the most weakly bound state. Binding stoichiometries at saturation for the two-headed Nkin460GST construct corresponded to two heads per MT heterodimer, as measured previously for two-headed ncd, Eg5 and ukinesin constructs. This stoichiometry is consistent with binding of one head only to each heterodimer of the MT, with the
Nkin walking mechanism

Fig. 2. Single MT viewed in video-enhanced DIC gliding over a single Nkin460GST molecule adsorbed to the surface of the coverslip. In the sequence (A–G) the MT rotates 180° whilst moving over a single point. The rotation point is marked ‘x’, the direction of movement is indicated by the arrow. Once the motor reaches the end of the MT (H), the MT diffuses away. The ATP concentration was 10 μM. The Nkin motor takes 40 s to walk the full length (1.3 μm) of the MT.

Table II. Estimated dissociation constants and binding stoichiometries for Nkin360 and Nkin460GST (binding to a fixed concentration of taxol-stabilized MTs)

<table>
<thead>
<tr>
<th></th>
<th>No added nucleotide</th>
<th>Mg-AMPPNP</th>
<th>Mg-ADP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μM)</td>
<td>(μM)</td>
<td></td>
</tr>
<tr>
<td>Nkin360</td>
<td>0.249</td>
<td>0.10</td>
<td>1.82</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>1.66</td>
<td>1.68</td>
<td>2.02</td>
</tr>
<tr>
<td>Nkin460GST</td>
<td>0.07</td>
<td>0.09</td>
<td>0.30</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>1.83</td>
<td>1.7</td>
<td>1.75</td>
</tr>
</tbody>
</table>

*aThe experiment was done in the presence of ATP, but considering the rate of ATP turnover the data reflect the analysis of the ADP state. The stoichiometry is expressed as head of motor per heterodimer of MTs.

other head held clear of the MT and retaining its ADP, as indicated by the kinetic data (see below). Remarkably, binding stoichiometries for the monomer construct Nkin360 also corresponded to two heads per MT heterodimer. The Nkin360 construct behaves like a monomer in solution, biophysically and kinetically; however, in this assay approximately two motor heads bind per MT heterodimer, suggesting that MT binding can provoke dimerization of Nkin360.

MT sliding assays
All of the expressed proteins (except Nkin360), including the GST fusions of the monomeric constructs, drove MT sliding in multi-molecule surface sliding assays. Rates were measured using RETRAC (see Materials and methods), and data are summarized in Table I. MT sliding also occurred on very low-density surfaces of double-headed Nkin. At low ATP concentrations, when sliding was slow, appreciable rotation of the sliding MT occurred about the single motor–MT link. The rotation was centred about a null (nodal) point (Hunt and Howard, 1993) (Figure 2). Under sliding at low ATP concentrations, it was possible to force rotation by pushing the MT using a bead gripped in the steerable optical trap. In this way the range of rotation could be increased, but was still narrower than that reported for full-length native ukinesin driving MT sliding over casein-coated glass (Hunt and Howard, 1993), being maximally only ~180° (Figure 2). We suspect that this relates to the truncation of our constructs relative to the full-length Nkin. Full-length Nkin, like full-length native ukinesin, has a number of short Pro,Gly-rich inserts in the tail (variously termed hinges or linkers), which could contribute additional torsional flexibility.

Nkin step size and stall force
Using the custom-built optical trapping microscope (see Materials and methods) we measured the step distance and stall force for single molecules of Nkin-His attached to 0.75 μm latex beads. The beads were captured out of solution using the laser trap, the trap was translated to the MT and parked, and the motion of the bead was recorded. The beads made repeated excursions from the trap centre (Figure 3A). The majority of transients attained a stall force of 4–5 pN. Even at high ATP concentration, the motor would sometimes hold stall force for a second or more before detaching.

How do we know these represent single molecule phenomena? For reasons that are unclear at present, capture of the His-tagged motor by the Ni beads was slow and/or inefficient, and motor was accordingly added in ~50-fold excess over the beads. At this ratio, and following rinsing, ~50% of the beads in the preparation were active. The best evidence that the observed interactions represent single molecules comes from the transients themselves. Most of the transients reach a plateau, and the plateaux are all at ~5 pN; in this sense the behaviour is quantal. In Figure 3A the majority of events reach 4–5 pN force...
and detach in a single unbinding event. At higher motor
densities, higher stall forces are achieved, and detachment
typically occurs in multiple unbinding events as multiple
motors detach sequentially from the MT (data not shown).
The long delay between events (10–20 s in Figure 3A)
is also consistent with the lengthy diffusional correlation
time required for a freely rotating bead to bring a single
motor molecule back into contact with the MT.

At low ATP concentrations and in favourable records,
individual steps could be resolved (Figure 3B, arrows).
The unitary step distance was close to 8 nm. Steps of
16 nm were seen rarely, consistent with their resulting
from summation of two concurrent 8 nm steps, each
occurring stochastically. At low loads the bead velocity
was as high as 1.5 μm/s, consistent with the 1.5–2.0 μm/s
observed in multimolecule surface sliding assays (Table I).

**MT-activated ATPase**

The basal ATPase of purified native Nkin heavy chain in
solution is ~0.05 s⁻¹ and is doubled in the presence of
MTs (Steinberg and Schliwa, 1996). Using the recombinant
two-headed Nkin460GST construct and the single-headed
Nkin360, we found that the basal level of ATPase (0.022
and 0.014 s⁻¹, respectively) was increased three orders of
magnitude in the presence of MTs, to 77.9 and 92 s⁻¹
per head at steady-state (Table III). The MT-activated ATPase
is unusually sensitive to temperature (not shown).
Nkin460GST MT-activated ATPase was not affected by
the presence of salt, but the single-headed construct
Nkin360 showed a marked increase in the K_{50% MTs}
in the presence of even a low concentration of NaCl (25 mM).

**Transient kinetics of stepping**

In the absence of MTs, ADP is essentially trapped in the
Nkin active site. Since MT binding efficiently triggers
ADP release, it is possible to use the release of the
fluorescent analogue mantADP to sense MT binding.
All the following experiments start with Nkin+mantADP
complexes in which the mantADP fluorophore is trapped
in the active site(s). Initial experiments indicated that
mixing such complexes with MTs in the presence of
different ‘chasing’ nucleotides released mantADP at differ-
et rates, depending on the species of chasing nucleotide,
with the amplitude of the fluorescence decay remaining
constant. Chasing mantADP from the motor active sites
using MTs and ATP, or the slowly hydrolysable analogue
AMPγS, gave apparently monophasic mantADP release
signals of 36 and 20 s⁻¹, respectively (Figure 4A, B, G,
and Table III). Chasing mantADP from the motor using
ADP or AMPPNP gave biphasic signals, each phase
corresponding to half the total amplitude (Figure 4C, D,
E, F and Table III). The faster process had a rate constant
of 43 s⁻¹ for AMPPNP and 35 s⁻¹ for ADP, whilst the
slower process had a rate constant of 4.9 s⁻¹ for AMPPNP
and 4.5 s⁻¹ for ADP.

The biphasic nature of some of the data suggested that
MT-activated ADP release occurs first from one head,
and then from the other. We suspected that mantADP release
occurred rapidly from the first head, at a rate independent
of the species of chasing nucleotide, but that the rate of
release of mantADP from the second head depends heavily
on the species of chasing nucleotide. Previous work with
kinesin has suggested this (Ma and Taylor, 1997; Gilbert
et al., 1998). The monophasic mantADP loss observed
for ATP and ATPγS chases would be consistent with
sequential release of mantADP from the first and second
heads, but at similar rates, such that the two processes
merge into a single phase. To test this interpretation, we
isolated the second phase of the biphasic mantADP release
signal, by mixing the motor•mantADP complex with
MTs before adding the chasing nucleotide. The transients
obtained from this experiment had approximately half the
amplitude observed in the first set of experiments, and
were well fitted by single exponentials with rate constants
consistent with the above interpretation (Figure 4B, D, F,
H and Table III). In these experiments also, the rates of
release of mantADP from the second head depended on
the chasing nucleotide used. An ATP chase released second
head mantADP at 60 s⁻¹, an ATPγS chase at 27 s⁻¹, an

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**Table III. Enzymatic features of the two constructs obtained by steady-state assay and transient assay**

<table>
<thead>
<tr>
<th></th>
<th>Steady-state</th>
<th>MT-activated ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Basal ATPase</td>
<td>MT-activated ATPase</td>
</tr>
<tr>
<td>Nkin460GST</td>
<td>0.022 s⁻¹</td>
<td>77.9 s⁻¹</td>
</tr>
<tr>
<td>Nkin360</td>
<td>0.014 s⁻¹</td>
<td>92 s⁻¹</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Transient</th>
<th>ATP</th>
<th>ATPγS</th>
<th>AMPγS</th>
<th>ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nkin460GST (No premix)</td>
<td>36.1 s⁻¹ (0.24)</td>
<td>20 s⁻¹ (0.27)</td>
<td>42.8 s⁻¹ (0.06)</td>
<td>35.5 s⁻¹ (0.10)</td>
<td></td>
</tr>
<tr>
<td>Premix</td>
<td>60.8 s⁻¹ (0.15)</td>
<td>26.8 s⁻¹ (0.12)</td>
<td>4.6 s⁻¹ (0.12)</td>
<td>3.0 s⁻¹ (0.10)</td>
<td></td>
</tr>
<tr>
<td>Nkin360 (No premix)</td>
<td>53.6 s⁻¹ (0.20)</td>
<td>50.1 s⁻¹ (0.21)</td>
<td>56.6 s⁻¹ (0.18)</td>
<td>50.2 s⁻¹ (0.17)</td>
<td></td>
</tr>
</tbody>
</table>

(A) Steady-state measurement of MT-activated ATPase rates from Nkin460GST and Nkin360.
(B) Rate of mantADP release from the motors Nkin460GST and Nkin360. The rates were obtained by fitting data to single or double exponentials to the transients corresponding to the decrease of fluorescence on loss of mantADP. The numbers in parentheses correspond to the amplitude of the signal (Volts); values can be compared for the same construct, but not between different constructs.

No premix, was obtained by labelling of the motor with mantATP, then chasing the mantADP from the motor by MT in the presence of nucleotide. Premix, was obtained by labelling of the motor with mantATP, then incubating with MT for 5 min and then chasing the mantADP from the motor with different nucleotides.
Fig. 3. Optical trap recordings from single Nkin-His molecules on Ni-NTA-coated latex beads. The traces show bead displacement in the direction of the MT (upper A and B) and perpendicular to the MT (lower A and B). Data were filtered with a 51 point (10 ms) averaging filter. The trap stiffness is 0.025 pN/nm in both traces. (A) 2 min of bead position data at 2 mM ATP are shown. In (B), at 20 μM ATP, walking progress is sufficiently slow to allow single steps to be resolved (arrows).

AMPPNP chase at 4.6 s⁻¹ and an ADP chase at 3 s⁻¹. The ADP result is especially informative (see Discussion).

The data argue for a sequential release model only if mantADP release from the first head of a pair of coupled heads does not depend on the species of chasing nucleotide. To check this, we mixed Nkin single heads (Nkin360) with MTs in the presence of each nucleotide. mantADP was then quantitatively released in a single, rapid process at ~57 s⁻¹ for an AMPPNP chase, 54 s⁻¹ for ATP, 50 s⁻¹ for ATPγS and 50 s⁻¹ for ADP. The rate of MT-activated mantADP release from single Nkin heads thus is essentially independent of the species of chasing nucleotide (Table III).

Discussion

**Single molecule evidence that Nkin is processive**

Using optical trapping nanometry, we found that single Nkin molecules execute runs of multiple steps against a retroactive optical force, confirming that Nkin is mechanic-
studied. Measurements of $K_d$ (Table II) for analogues of intermediates in ATP turnover were consistent with a scheme in which, as for other kinesins, the motor-ADP state is the weakest binding state in the conformational cycle, MT binding activates ADP release from the motor by ~10$^{-2}$-fold and MT-activated ADP release corresponds to a switch from weak to strong binding. As with ukin, binding of two-headed Nkin to MTs released half of the bound mantADP at a rate independent of the species of chasing nucleotide, and the other half at a rate that depends on the species of chasing nucleotide. The effectiveness of the various chasing nucleotides was different for Nkin compared to ukin (Ma and Taylor, 1997; Gilbert et al., 1998), suggesting differences between the kinetic coordination mechanisms of the two motors. For Nkin, binding of the slowly hydrolysable analogue AMPPNP to the trailing head released mantADP from the leading head at 4.6 s$^{-1}$. Since AMPPNP generates a tightly bound complex of motor and MT (Table II), we imagine that the rate of 4.6 s$^{-1}$ for mantADP release from the second head reflects the rate at which the second head can locate and bind to its next site. Binding the slowly hydrolysable analogue ATP$\gamma$S to the trailing head stimulates much faster (26.8 s$^{-1}$) mantADP release from the leading head, indicating that this hydrolysable nucleotide induces a conformational change of the trailing head that accelerates attachment of the leading head to the MT. Binding and hydrolysis of ATP itself stimulates the fastest mantADP release from the leading head (60 s$^{-1}$). ATP is hydrolysed faster than ATP$\gamma$S, again consistent with a need for hydrolysis and/or $P_i$ release on the trailing head to induce the fastest leading head attachment.

Binding of ADP to the trailing head induces leading head ADP release at only 4.5 s$^{-1}$. This result is doubly informative. First, it is again consistent with the idea that for Nkin, ATP hydrolysis on the trailing head is needed for rapid leading head attachment. Secondly, the finding that with ADP on the trailing head, leading head attachment occurs at only 4.5 s$^{-1}$ also implies that in the normal cycle, trailing Nkin•ADP heads ordinarily do not detach spontaneously. To see this, consider that leading head attachment could, in principle, occur by two different pathways (Figure 5). In the mechanically processive pathway (Figure 5A), the trailing head remains attached on the timescale of the experiment, and the leading head reaches forward, binds, and loses its mantADP by MT-activated mantADP release. In the detaching pathway (Figure 5B), ADP binding to the trailing head induces it to detach, and mantADP release by the leading head is due to repeated cycles of detachment and rebinding of the whole molecule, with each cycle leading to loss of mantADP from half the population of Nkin•mantADP heads by MT-activated mantADP release. If for the detaching pathway we set the rate constant for MT-activated mantADP release via the landing reaction to 40 s$^{-1}$ (the same as the measured rate of mantADP release from the leading head following mixing of Nkin•mantADP with MTs), then to account for the observed rate of mantADP release of only 4.5 s$^{-1}$, the rate of spontaneous detachment of Nkin•ADP trailing heads would need to be <10 s$^{-1}$. Thus, if a processive scheme applies, Nkin–ADP trailing heads must detach at 4.5 s$^{-1}$ or less, and if a detaching scheme applies, Nkin•ADP trailing heads must

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**Kinetic coordination of Nkin stepping**

Our current data indicate that the Nkin ATPase mechanism is broadly similar to that of all other kinesins so far
detach at 10 s\(^{-1}\) or less. Whichever pathway applies, we can make the general inference that Nkin•ADP trailing heads detach spontaneously at 10 s\(^{-1}\) or less. This is a crucial result; the rate is at least 8-fold slower than the rate of ATP turnover, which under these conditions is 78 s\(^{-1}\). It follows that if the detachment of Nkin•ADP trailing heads is to be part of the normal mechanochemical cycle, the trailing heads must routinely be pulled off; their rate of spontaneous detachment is otherwise too slow to allow stepping at 78 s\(^{-1}\) per head. If Nkin•ADP trailing heads are not pulled into their detaching trapped ADP conformation, they will undergo MT-activated ADP release and start a new cycle of ATP turnover, leading to futile cycling (see below).

**Mechanochemical model**

The mechanical data indicate that single molecules of Nkin, like those of ukinesin, move along MTs in 8 nm steps, and make progress against retroactive optical forces of up to 5 pN. The chemical kinetic data suggest how this stepping action is coordinated. Coordinated stepping depends on the motor being able to identify consistently and detach its trailing head. We find at least a 15-fold acceleration (4 to 60 s\(^{-1}\)) of the rate of attachment of Nkin•ADP leading heads following ATP hydrolysis on the trailing head, and at least a 6-fold acceleration of trailing Nkin•ADP head detachment (<10 to 60 s\(^{-1}\)) following ADP release from the leading head. Figure 6 builds these two mutual dependencies into a scheme for the mechanochemical cycle. The data provide direct experimental support for the major features of this scheme, which would operate as follows: upon initial binding to the MT, ADP is rapidly released from one head only, whilst the other head remains in the Nkin•ADP state, poised but initially unable to access the MT. Sequential ATP binding, hydrolysis and possibly Pi release by the trailing head then stimulates leading head attachment and coupled MT-activated ADP release. This release of ADP by the leading head then, in turn, accelerates the otherwise slow transition of the trailing head into its detaching (weak-binding) Nkin•ADP conformational state. Effectively, the trailing head is pulled off by the attachment of the leading head, and the cycle begins again.

**Coupling efficiency**

In this scheme (Figure 6), the leading head remains poised, trapped in the Nkin•ADP state and unable to access the MT until ATP is bound and hydrolysed by the trailing head. ATP turnover on the leading head is thus tightly coupled to that on the trailing head. The nature of the other coupling, between leading head attachment and the release of the trailing head, is less clear. By the argument given in the Results section, we know that there is a coupling such that leading head attachment accelerates trailing head detachment at least 6-fold, presumably by pulling on the trailing head. This coupling will be tight only if the rate at which the trailing heads are pulled off is high relative to the rate at which trailing heads cycle through to ADP release and bind a fresh ATP without detaching. A side-observation indicates that this coupling may be variably efficient, in other words that futile cycles of ATP turnover may occur. We noticed that Nkin in multimolecule sliding assays exhausts the ATP supply very rapidly compared with ukinesin in analogous assays, suggesting that crowding on the MT may indeed produce futile kinetic cycles. It thus seems possible that whilst leading head attachment requires (is tightly coupled to) ATP binding and hydrolysis by the trailing head, multiple cycles of ATP turnover may nonetheless occur (loose...
coupling) if the trailing head is not pulled off by the leading head. The scheme shown (Figure 6) incorporates this property, in that a slow step prior to ADP release by the trailing head is accelerated by forwards strain originating from the attachment of the leading head, but nevertheless, ADP release by the trailing head can occur spontaneously (under no strain) at a significant rate. The effect of this would be to cause the motor to retry leading head attachment several times before detaching both heads and aborting a processive run, allowing progress to be resumed if an obstacle were only temporary, at the cost of a modest number of futile cycles of ATP turnover.

Towards a general model for processivity in kinesins

Here we have identified Nkin as a second mechanically processive kinesin, and based on transient kinetic data have worked out a coordination scheme that shows two apparently new features: (i) that trailing head ATP hydrolysis is necessary for rapid leading head attachment; and (ii) that during stepping the trailing Nkin–ADP head of each molecule is pulled by the leading head into its detaching conformation. Studies of ukinesin have already found coupling between ADP release on the leading head and events on the trailing head, but in these studies AMPPNP-induced leading head ADP release was only 3-fold slower than that induced by ATP. Based principally on this finding it was suggested that ATP binding, rather than a subsequent step in ATP turnover, ordinarily triggers leading head attachment. For Nkin, AMPPNP is 15-fold less efficient than ATP, and it is more obvious that hydrolysis accelerates leading head attachment. Nonetheless, the difference in efficiency between AMPPNP and ATP is still there in the ukinines data. For both ukinines and Nkin, ATPγS, a slowly hydrolysed analogue, induced second head ADP release at an ~3-fold slower rate than that induced by ATP. There are no data on the relative rates of ATPγS hydrolysis by the two motors, but the result nonetheless suggests that the differences between the mechanisms are only subtle. Likewise, for both motors, AMPPNP does accelerate second head binding, but simply at different efficiencies. Accordingly, the data suggest a unifying model in which for both motors, each step in ATP turnover progressively favours attachment of the leading head, but with subtle differences between Nkin and ukin in the incremental contributions of each chemical step. The data strongly support the idea that kinesin heads can communicate via mutual mechanical strain. An important technical challenge now is to measure the kinetic effects of mechanical strain on single kinesin molecules.

Materials and methods

Construction of Nkin360, Nkin360GST and Nkin460GST

For all the constructs the same forward primer was used: 5'-GTCCATATGTCGAGAATTCCTGGAAGGACGTACGGTCTTTGCCTT-3'. This forward primer introduced an Ndel site (underlined) onto the 5' end of the DNA. The following reverse primers were used: 5'-CACATAGTT-GAATTCCTGGAAGGACGTACGGTCTTTGCCTT-3' was used to amplify the 1080 bp clone Nkin360, and 5'-CACATAGTTGA-ATTCAGCCGCGCCGATCTTTCTTCCG-3' was used to amplify the 1380 bp clone Nkin460. These reverse primers introduced an EcoRI site and a SpeI site (underlined) onto the 3' end of the DNA. PCRs were performed using the proof-reading, thermostable DNA polymerase Vent (New England Biolabs), and used 20 cycles of amplification to minimize the chance of random errors in the PCR-generated gene. The amplified DNA was purified from an agarose gel, digested with Ndel–SpeI and ligated into Ndel–SpeI-cut pET17b. The plasmids were termed pETNkin360 and pETNkin460. To engineer the GST fusion constructs the cloning was continued as follows. Plasmids were digested with EcoRI and ligated to a 660 bp DNA fragment encoding the GST gene with EcoRI sticky ends and an in-frame stop codon at the 3' end to create the plasmids pETNkin360GST and pETNkin460GST.

Expression and purification of the constructs

Expressions were performed in BL21 (DE3) cells freshly transformed with the plasmids. Overnight cultures of bacteria were diluted 1:100 into 2× YT medium supplemented with ampicillin (100 μg/ml) and grown, with shaking, at 37°C until the absorbance at 600 nm was 1. The cells were shaken for a further 30 min at 30°C before induction with isopropyl β-D-thiogalactopyranoside (0.25 mM), and after a further 4 h shaking at 30°C the bacteria were harvested by centrifugation (typically 8 × 1 l) shaker cultures were grown). The cell pellets obtained were flash-frozen in liquid nitrogen and stored at –80°C.

Purification of proteins

The cell pellets were resuspended in the buffers stated below, and for all purifications the solution was supplemented with Complete Protease Inhibitor Cocktail tablets (Boehringer Mannheim) at the recommended dosage and incubated on ice with lysozyme (0.1 mg/ml) for 20 min. The cell lysate was supplemented with Triton X-100 (0.05%) and deoxyribonuclease I (40 μg/ml), and incubated for a further 10 min on ice. The lysate was clarified by centrifugation (27 000 g, 50 min, 4°C) and the cell pellet discarded. All chromatography steps were performed using an FPLC system (Pharmacia Biotech, St Albans, UK) at 4°C. Column fractions were analysed for purity by SDS–PAGE (Laemmli, 1970) and the peak fractions pooled. Frozen protein stocks were prepared by supplementing the purified protein solution with 20% glycerol, flash-freezing and storing aliquots in liquid nitrogen. When stored in this manner the protein was stable indefinitely, and once thawed was used immediately. Concentrations of motor are expressed throughout as concentrations of active sites (there is one active site per head).

GST constructs

Cell pellets were resuspended in phosphate-buffered saline containing 1 mM diithiothreitol (DTT), 5 mM MgCl₂ and 10 μM ATP. Nkin460GST was purified by passing the clarified supernatant of the cell lysate over a 10 ml gluthatione Sepharose 4B column equilibrated in buffer A (20 mM MOPS pH 7.2, 1 mM DTT, 5 mM MgCl₂, 10 μM ATP). After extensive washing of the column with buffer A, bound protein was eluted with buffer B supplemented with 20 mM reduced glutathione and 50 mM NaCl. The eluted fusion protein was then diluted 1:1 in buffer A and applied to a 5 ml High Trap S column equilibrated in buffer A plus 50 mM NaCl. Nkin460GST was step-eluted from this column using buffer A supplemented with 200 mM NaCl. The concentration of Nkin460GST was determined using a calculated extinction coefficient of 71240 M⁻¹ cm⁻¹. Nkin360GST was prepared in the same way as Nkin460GST. The calculated extinction coefficient was 58580 M⁻¹ cm⁻¹.

Purification of Nkin-His

The construct (pT7-7/Nkin-His₅₀) coding for full-length Nkin + His₅₀ (Nkin-His), was a kind gift from M.Wagenbach (J.Howard laboratory); it was expressed as the other constructs. Cell pellets were resuspended in 20 mM MOPS buffer pH 7.2 containing 5 mM β-mercaptoethanol, 5 mM MgCl₂, 100 mM NaCl and 10 μM ATP. Nkin-His was purified by passing the clarified supernatant of the cell lysate over a 10 mlgluthatione Sepharose 4B column equilibrated in buffer A and applied to a 5 ml High Trap S column equilibrated in buffer A plus 50 mM NaCl. Nkin-HisGST was step-eluted from this column using buffer A supplemented with 200 mM NaCl. The concentration of Nkin-HisGST was determined using a calculated extinction coefficient of 35940 M⁻¹ cm⁻¹.

Purification of Nkin360

Cell pellets were resuspended in 20 mM sodium phosphate buffer pH 7.4 containing 1 mM DTT, 5 mM MgCl₂ and 10 μM ATP. Nkin360 was purified by passing the clarified supernatant over a 5 ml Hitrap S column equilibrated in buffer B (20 mM sodium phosphate pH 7.4, 1 mM DTT, 5 mM MgCl₂, 10 μM ATP). After extensive washing of the column with buffer A, Nkin360 was step-eluted from the column using buffer B supplemented with 50 mM NaCl. The concentration of Nkin360 was determined using a calculated extinction coefficient of 16580 M⁻¹ cm⁻¹.
For Nkin360 and Nkin460GST, N-terminal micro sequencing from Western blots was performed to check the N-termini were intact. The sequencing was performed at the University of Newcastle Molecular Biology Unit, and returned the sequence SSSAN for all the bands assayed.

**Estimation of Stokes’ radii**

The Stokes’ radii ($R_s$) of Nkin360 and Nkin460GST were determined using a Superose 12 (Pharmacia) gel filtration column equilibrated in 20 mM MOPS pH 7.2, 2 mM DTT, 2 mM MgCl$_2$, 10 μM ATP. The column was calibrated with proteins of known $R_s$: aldolase ($R_s = 4.81$ nm), bovine serum albumin (BSA) ($R_s = 3.55$ nm) and chymotrypsinogen ($R_s = 2.09$ nm).

**Estimation of sedimentation coefficients**

Sedimentation coefficients ($S_{20w}$) were determined by rate zonal centrifugation on a Beckman L80.001 Ti rotor at 60 000 r.p.m. in a Beckman UV-1201 spectrophotometer connected to a PC. A stable baseline was recorded first and then the motor was added to the assay mixture.

The concentration of motor proteins used in the assays was kept as low as possible in order to minimize the reported crowding effects on MTs, (Huang and Hackney, 1994). Typically, 0.060 μM Nkin460GST and 0.225 μM Nkin360 were used as final concentrations in the assay. For the measurement of basal ATPase 5 μM motor was used.

**Stopped flow analysis**

Rapid mixing experiments were performed with a Hitech SFX61 spectrofluorimeter (HiTech Scientific, Salisbury, UK). MT concentrations were assaying for the ATPase, based on steady-state assays of MT-activated ATPase. These were 0.5 μM motor, 10 μM ATP, 20 and 30 μM MT for experiments using Nkin460GST and Nkin360, respectively. The final composition of the reaction buffer was 20 mM MOPS pH 7.2, 1 mM DTT, 5 mM MgCl$_2$, 1 mM nucleotide (ATP, AMPPNP, ATP$_8$, ADP). In addition, 25 mM NaCl was added for experiments with Nkin460GST. Nkin460GST or Nkin360 were labelled with mantADP by incubating 1 μM motor with 4 μM mantATP at 21 °C for 15 min. To observe the decrease of fluorescence corresponding to MT-activated release of mantADP from the active site, we set up two different types of experiment. In one, termed ‘no premix’, the first syringe of the stopped flow apparatus contained labelled motor, and the second syringe contained MTs and the chasing nucleotide. In the other, termed ‘premix’, the labelled motor was incubated and then loaded into the first syringe. The second syringe contained only the chasing nucleotide. All data were obtained from three pushes on at least two different days.

**Optical trapping apparatus**

Single molecule events were recorded on an optical trapping microscope based on a Zeiss Axiosvert 135TV. The trapping laser was a 3 W CW NdYAG (Uniphase STA 1064-3E, CW). The optical trap was moved by steering the laser using two orthogonally mounted acousto-optic deflectors (AODs; supplied by Isis Optics, UK) in the arrangement described by Molloy et al. (1995). The objective lens was the Zeiss 100×, 1.25NA oil immersion. A standard Zeiss 100 W tungsten/halogen lamp house was used for illumination. A custom-made condenser (using 40×, iris, 1.3NA Zeiss oil immersion objective) enabled the observation of single MTs in real time using video-enhanced DIC (Hamamatsu, Argus 20). Because of the short working distance of this condenser, microscope samples were made up in a flow cell using two coverslips. Coarse stage position was adjusted using a micromanipulator (Physik Instrumente, M-312.00) and fine alignment was carried out by a feedback-controlled piezoelectric XY stage (50 × 50 μm movement) with sub-nanometre resolution and repeatability (Physik Instrumente, P-730.20). A stepper motor (400 steps/rev. in half step mode) controlled the focus. Backlash compensation was done in the software. Trapped bead position was measured using brightfield optics (by removing both the polarizing filters used for DIC). The bead image was projected onto a four-quadrant photodiode (Hamamatsu, S1-P40-43) allowing the bead position to be measured in two axes (XY) with sub-nanometre accuracy. The detector circuit was modified from the design of Simmons et al. (1996). The roll-off frequency of the position detector and associated circuitry was 8.5 kHz. The linear region of this detector extended to ± 200 nm in both axes. Bead position (X and Y) data were digitized at 40 kHz (PC data acquisition card PCL-1800, Advantech Co., Ltd) and this data was averaged down to 5 kHz in real time in software. The target MT position (XY and Z) and orientation were recorded with the aid of a real time video overlay. The orientation information was used to convert the XY bead position data into ‘on-axis’ (axial with the MT) and ‘off-axis’ channels in real time. With this data rotation, recordings could be made on MTs at any orientation on the coverslip surface. Data traces were saved to disk in the unfiltered XY format along with the MT orientation information and apparatus calibration data. Software written by N.J. Carter controlled microscope focus, stage position, trap position, laser power, trap position, data acquisition, frame grabbing, video overlay, real time data filtering/plotting and all analysis functions.

**MT pelleting assays**

The pelleting assays were performed according to Lockhart et al. (1995). Increasing amounts of motor (1–4 μM bead) in buffer D were mixed with a constant amount of MTs (2.8 μM heterodimer in buffer D) in the presence of 1 mM ATP, 1 mM AMPPNP or no nucleotide. The samples (40 μl) were gently mixed then centrifuged in a Beckman TLX 100 ultracentrifuge (100 000 g, 10 min, 25°C). The supernatants were carefully aspirated, and the MT pellet carefully washed with BRB80 (80 mM K–PIPES pH 6.9, 1 mM MgCl$_2$, 1 mM EGTA, 0.2 mM MgCl$_2$, 0.01 mM GTP and 20% glycerol) was polymerized by the addition of MgCl$_2$ and GTP to final concentrations of 2 and 1 mM, respectively. The tubulin was incubated for 30 min at 37°C, at which point paclitaxel (taxol) was added to a final concentration of 20μM. In order to remove guanine nucleotides, the taxol-stabilized MTs were pelleted by centrifugation (100 000 g, 10 min, 25°C), the supernatant was aspirated, and the MT pellet carefully washed with BRB80 (80 mM K–PIPES pH 6.9, 1 mM MgCl$_2$, 1 mM EGTA) containing 20 μM taxol. For motility assay, the MTs were resuspended in this same buffer. For the steady-state ATPase assays the MTs were suspended in buffer C (20 mM MOPS pH 7.2, 1 mM DTT, 5 mM MgCl$_2$, 0.1 mg/ml BSA and 20 μM taxol). For transient kinetic measurements and pelleting assays MTs were suspended in buffer D (20 mM MOPS, 1 mM DTT, 5 mM MgCl$_2$, 25 mM NaCl, 20 μM taxol). All MT concentrations are expressed per tubulin heterodimer.

**Measurement of steady-state ATPase rate**

MT-activated ATPase rates were determined using a pyruvate kinase/ lactate dehydrogenase-linked assay. The assays were performed in 350 μl reaction volumes using buffer B. Phospho(enol)pyruvate, NADH and Mg-ATP were added to final concentrations of 3, 0.2 and 1 mM, respectively, along with 14 U of pyruvate kinase, 22 U of lactate dehydrogenase and MTs resuspended in buffer D with or without NaCl. All assay reagents were from Sigma. The absorbance of NADH was monitored at 340 nm in a temperature-controlled (21°C) Shimadzu UV-1201 spectrophotometer connected to a PC. Stable baseline was recorded first and then the motor was added to the assay mixture.

The concentration of motor proteins used in the assays was kept as low as possible in order to minimize the reported crowding effects on MTs, (Huang and Hackney, 1994). Typically, 0.060 μM Nkin460GST and 0.225 μM Nkin360 were used as final concentrations in the assay. For the measurement of basal ATPase 5 μM motor was used.

**Nkin walking mechanism**

The concentration of motor proteins used in the assays was kept as low as possible in order to minimize the reported crowding effects on MTs, (Huang and Hackney, 1994). Typically, 0.060 μM Nkin460GST and 0.225 μM Nkin360 were used as final concentrations in the assay. For the measurement of basal ATPase 5 μM motor was used.
Block, I.M., Lockhart, A. and Cross, R.A. (1997) Kinetic evidence for low μ('BRB-supp'). In assays where the ATP concentration was below 100 μM, an ATP regenerating system was used (50 μg/ml creatine phosphokinase and 1 mM phosphocreatine). Flow cells were made using two ethanol-wetted coverslips (20 × 22 and 22 × 22 mm, 2B1) sealed using silicon grease (Dow Corning). The larger coverslip was coated with a layer of nitrocellulose. The Nkin motor was washed into the flow cell at various concentrations (0.1–10 μM, depending on motor density required) along with 100 μg/ml casein as surface block. Motor was allowed to adsorb to the surface for 5 min at room temperature. Non-adsorbed motor was washed out with 2–3 flow cell volumes of BRB-supp. Flow cell volumes were between 5 and 10 μl. Two flow cell volumes of MTs in BRB-supp with the desired concentration of ATP were then flowed through. All motility assay recordings were made at a controlled 23.5°C. The measurement of MT velocities was performed using N.J.Carter’s freeware RETRAC program (http://mc11.mcri.ac.uk/retrac) for image capture and tracking.

Optical trapping flow cell preparation

All solutions were based on `sol A`, which contained 80 mM PIPES pH 7.0, 1 mM MgCl₂, 0.1% β-mercaptoethanol, 10 μM taxol and 2 mM phosphocreatine. Polystyrene beads (0.75 μm diameter, Polysciences, Inc., Cat #07309) with very low densities of adsorbed motor were prepared as follows. The beads were washed twice by vortexing followed by centrifugation in sol A. The beads were resuspended in sol A with 2 mg/ml F108-NTA and 10 mM NiCl₂ (deCastro et al., 1999). After 30 min of NTA adsorption time the beads were centrifuged, washed three times, and resuspended in sol B, a casein block solution (sol A with 100 μg/ml casein). The Nkin-His motor was added at a concentration of 1 μM or less (beads are ~0.02 μM at this stage). After 10 min on ice the beads were centrifuged, washed twice, and resuspended in sol B. MTs were washed into the flow cell and allowed 10 min adsorption time at room temperature. The MTs still in solution were washed out with 2–3 flow cell volumes of sol B. After 5 min, the beads were washed in at very low densities in sol B with the desired ATP concentration and an oxygen scavenging system (3 mg/ml glucose, 100 μg/ml glucose oxidase and 20 μg/ml catalase). If necessary the bead preparation method was repeated, from the motor addition stage, reducing the amount of motor added until motor loading was such that approximately half of the beads would neither run in the presence of ATP nor bind to MTs in zero ATP conditions (rigor). All recordings were made on MTs that were firmly attached (i.e. no visible Brownian movement) to the lower, larger coverslip. Using the larger flow cell and allowed 10 min adsorption time at room temperature. The MTs which were between 5 and 10 μM were between 5 and 10 μM. Two flow cell volumes of MTs in BRB-supp with the desired concentration of ATP were then flowed through.