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-methylanthraniloyl) 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 ATPγS 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, 1999; 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).

Fig. 1. Top, greyscale ccd images of Coomassie Blue-stained SDS gels from MT pelleting assays. Left panels, Nkin360, and right panels, Nkin460GST, were incubated in the presence of 2.8 μM MT heterodimers. Pell, pellets (5 μl sample loaded); Sup, supernatants (5 μl of sample loaded), with the exception of the first three points where 7.5 μl of sample (or 10 μl for Nkin460GST AMPPNP) were loaded. Bottom, corresponding binding isotherms for Nkin360 (left) and Nkin460GST (right). Motor and MT concentrations are expressed per motor domain and per tubulin heterodimer. Dissociation constants and binding stoichiometries were estimated by fitting the data to rectangular hyperbolae (solid line) see Table II; dashed lines correspond to one head (at 2.8 μM) or two heads (at 5.6 μM) of motor per MT heterodimer.

Table I. Physical properties and MT sliding velocities of Nkin460GST, Nkin360 and Nkin360GST

Constructs	R_s (nm)	(S_{20w})	Calculated molecular mass (kDa)	Polypeptide molecular mass (kDa)	Velocity multimotor assay ($\mu\text{m/s}$)	Association state
Nkin460GST	5.3	6.58	93.7	75.9	1.8 ($\pm$ 0.2)	dimer
Nkin360	3.1	1.61	27.5	39.3	not detected	monomer
Nkin360GST	4.9	6.17	84.5	64.8	0.15	dimer

Stokes' radii (R_s) were determined by gel filtration chromatography and the sedimentation coefficients (S_{20w}) by rate zonal centrifugation. The calculated molecular mass was determined according to Freifelder (1982), and the polypeptide molecular mass from the protein's primary sequence.

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 ukinesin•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

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 μ M. The Nkin motor takes 40 s to walk the full length (1.3 μ m) of the MT.

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

		No added nucleotide	Mg-AMPPNP	Mg-ADP ^a
Nkin360	K_d (μ M)	0.249	0.10	1.82
	Stoichiometry	1.66	1.68	2.02
Nkin460GST	K_d (μ M)	0.07	0.09	0.30
	Stoichiometry	1.83	1.7	1.75

^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 $\sim 180^\circ$ (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 μ m 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, $\sim 50\%$ 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

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

A	Steady-state	Basal ATPase		MT-activated ATPase	
	Nkin460GST	0.022 s ⁻¹	$V_{\max}$	$K_{50\%}$	
	Nkin360		77.9 s ⁻¹	0.21 μ M	
		0.014 s ⁻¹	$V_{\max}$	$K_{50\%}$	
			92 s ⁻¹ (95 s ⁻¹) ^a	3 μ M (11 μ M) ^a	
B	Transient	ATP	ATP γ S	AMPPNP	ADP
	Nkin460GST (No premix)	36.1 s ⁻¹ (0.24)	20 s ⁻¹ (0.27)	42.8 s ⁻¹ (0.06)	35.5 s ⁻¹ (0.10)
	Premix	60.8 s ⁻¹ (0.15)	26.8 s ⁻¹ (0.12)	4.9 s ⁻¹ (0.11)	4.5 s ⁻¹ (0.11)
	Nkin360 (No premix)	53.6 s ⁻¹ (0.20)	50.1 s ⁻¹ (0.21)	4.6 s ⁻¹ (0.12)	3.0 s ⁻¹ (0.10)
	Premix	0	0	56.6 s ⁻¹ (0.18)	50.2 s ⁻¹ (0.17)
				0	0

(A) Steady-state measurement of MT-activated ATPase rates from Nkin460GST and Nkin360.

^aCorresponding values in the presence of salt.

(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.

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 μ m/s, consistent with the 1.5–2.0 μ m/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 different 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 ATP γ S, gave apparently monophasic mantADP release signals of 36 and 20 s⁻¹, respectively (Figure 4A, B, G, H 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 ATP γ S 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 ATP γ S chase at 27 s⁻¹, an

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 μ M 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 $\sim 57 s^{-1}$ for an AMPPNP chase, 54 s^{-1} for ATP, 50 s^{-1} for ATP γ S 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-

Fig. 4. Kinetics of mantADP release from Nkin460GST. Representative fluorimeter traces in which Nkin460GST (0.5 μM) is labelled with mantATP (2 μM) and rapidly mixed with excess MT (20 μM heterodimer) plus 1 mM of the following nucleotides: (a) ATP; (c) ADP; (e) AMPPNP; and (g) ATP γ S (concentrations are those in the flow cell after mixing). Lower graphs, same final concentrations but the experiments were done differently; after labelling, the motor was mixed with MT, then the release of mantADP was followed when the motor•MT labelled complex was rapidly mixed with 1 mM of the following nucleotides: (b) ATP; (d) ADP; (f) AMPPNP; and (h) ATP γ S. The solid black line corresponds to the best fit of the data to either one (a, g, b, d, f and h) or two (c and e) exponentials (see Table III).

ally processive. Nkin stalls at a force of 4–5 pN, which is within the range reported for ukinesin (Svoboda *et al.*, 1993; Kojima *et al.*, 1997). Favourable Nkin records (Figure 3) showed discrete 8 nm steps, corresponding to the unitary step distance between adjacent MT heterodimers on the MT protofilament. We have seen no evidence so far that Nkin takes long (16 nm) steps more frequently than ukinesin, although further work will be necessary to be certain of this point. At saturating ATP concentrations, the velocity of Nkin (1.5–2 $\mu\text{m/s}$) was ~ 3 -fold faster than that for ukinesin (0.6 $\mu\text{m/s}$), both in single molecule assays and multi-motor surface sliding assays. Nkin motors thus share the remarkable ability of ukinesin to exert minimal mutual drag when working in teams, allowing movement at near-maximal velocity in multi-motor assays. In single-molecule surface sliding assays, long processive runs of up to several hundred 8 nm steps were observed.

In sliding assays at low motor density, we observed nodal point pivoting of the sliding MTs. The angular range of the pivoting was reduced compared with that reported for native ukinesin, suggesting that the recombinant Nkin motor is torsionally stiffer than native ukinesin. It is possible, however, that the apparently high torsional stiffness of Nkin may be a property only of the recombinant Nkin construct used here, which is shorter than the native protein.

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

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 $\sim 10^3$ -fold and MT-activated ADP release corresponds to a switch from weak to strong binding. As with ukinesin, 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 ukinesin (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 γ 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 γ 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 $< 10 \text{ s}^{-1}$. 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

Fig. 5. Schematic representation of the two possibilities of succession of events leading to loss of mantADP from both active sites of a dimeric construct, after encounter with MT, in the presence of ATP. (A) Processive scheme. (B) Non-processive scheme.

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 P_i 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

Fig. 6. Model of the mechanochemical coordination mechanism. The critical (most likely) path is illustrated. Binding of ATP (○) to the trailing head is not sufficient to induce rapid leading head attachment. The subsequent ATP hydrolysis step must be complete before rapid leading head attachment can occur. Attachment of the leading head causes it to release ADP, and this produces a sustained pull on the trailing head, accelerating its transition into the weak-binding Nkin–ADP state. The rate of phosphate release will govern the flux into this weak-binding Nkin•ADP state, and it is possible that the rate of phosphate release is sensitive to the strain exerted by the attaching leading head.

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 ukinesin data. For both ukinesin and Nkin, ATP γ S, 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 ATP γ S 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'-GTCCATAATGTCGTCAAGTGC GAATAGTATCAAGGTC-3'. This forward primer introduced an *NdeI* site (underlined) onto the 5' end of the DNA. The following reverse primers were used: 5'-CAACTAGTTGAATTCCTCGAAGGACGTGATCTGGGTCTTGGCCTT-3' was used to amplify the 1080 bp clone Nkin360, and 5'-CAACTAGTTGAATTCAGCCGCCGCGATACTTCTCTTCCG-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 *NdeI*-*SpeI* and ligated into *NdeI*-*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 $\times$ YT medium supplemented with ampicillin (100 μ g/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 $\times$ 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 μ g/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 diethiothreitol (DTT), 5 mM MgCl₂ and 10 μ M ATP. Nkin460GST was purified by passing the clarified supernatant of the cell lysate over a 10 ml glutathione Sepharose 4B column equilibrated in buffer A (20 mM MOPS pH 7.2, 1 mM DTT, 5 mM MgCl₂, 10 μ M ATP). After extensive washing of the column with buffer A, bound protein was eluted with buffer A 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⁻¹cm⁻¹. Nkin360GST was prepared in the same way as Nkin460GST. The calculated extinction coefficient was 58580 M⁻¹cm⁻¹.

Purification of Nkin-His

The construct (pT7-7/Nkin-His₆) coding for full-length Nkin + His₆ (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 μ M ATP. Nkin-His was purified by passing the clarified supernatant of the cell lysate over a 5 ml 'Talon' metal affinity column (Clontech). The column was equilibrated with 50 mM sodium phosphate pH 8, 5 mM MgCl₂, 100 mM NaCl, 5 mM β -mercaptoethanol, 10 μ M ATP. The column was then rinsed with the same buffer supplemented with 10 mM imidazole, and the protein was eluted from the column with the same buffer containing 100 mM imidazole. The calculated extinction coefficient was 35940 M⁻¹cm⁻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 μ M 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 μ M ATP). After extensive washing of the column with buffer A, Nkin360 was step-eluted from the column using buffer A supplemented with 50 mM NaCl. The concentration of Nkin360 was determined using a calculated extinction coefficient of 16580 M⁻¹cm⁻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, 200 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 10 μ M 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 15–35% (v/v) glycerol gradient in 20 mM MOPS pH 7.2, 1 mM DTT, 2 mM $MgCl_2$, 10 μ M ATP, 200 mM NaCl. The gradients were centrifuged at 50 000 r.p.m. in a TST60 rotor (Sorvall) for 21 h. Ferritin (13S), catalase (11.3S), rabbit muscle aldolase (7.3S), creatine kinase (5.3S) and BSA (4.6S) (all from Sigma) were run as standards. Plots of relative distance travelled by the standards versus their sedimentation coefficients yielded straight lines from which the sedimentation coefficients of both proteins were estimated.

Estimation of molecular masses

The molecular masses of Nkin460GST and Nkin360 were calculated by co-sedimentation with proteins of known molecular mass [ferritin (440 kDa), catalase (230 kDa), rabbit muscle aldolase (150 kDa), rabbit muscle creatine kinase (81 kDa), BSA (67 kDa) and bovine pancreas chymotrypsinogen (25 kDa) (Pharmacia)]. The gradients prepared as above were centrifuged at 50 000 r.p.m. in a TST60 rotor (Sorvall) for 21 h.

Plots of the relative distance travelled by the standards versus their log molecular masses yielded a straight line from which the molecular masses of Nkin460GST and Nkin360 were estimated according to the method of Freifelder (1982).

Purification and polymerization of tubulin

Tubulin was purified from porcine brain as described in Lockhart and Cross (1994). Tubulin (typically 5 mg/ml in 50 mM PIPES pH 6.9, 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 20 μ M. 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 BRB 80 (80 mM K-PIPES pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA) containing 20 μ M 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 μ M 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 μ M taxol). All MT concentrations are expressed per tubulin heterodimer.

MT pelleting assays

The pelleting assays were performed according to Lockhart *et al.* (1995). Increasing amounts of motor (1–4 μ M head) in buffer D were mixed with a constant amount of MTs (2.8 μ M heterodimer in buffer D) in the presence of 1 mM ATP, 1 mM AMPPNP or no nucleotide. The samples (40 μ l) were gently mixed then centrifuged in a Beckman TLX 100 ultracentrifuge (100 000 g, 10 min, 20°C). The supernatants were carefully aspirated, 10 μ l of SDS sample buffer was added, and the pellets resuspended in 40 μ l of 1M NaCl + 10 μ l of SDS sample buffer. Pellet and supernatant were run on SDS gel slab gels and stained with Coomassie Brilliant Blue greyscale ccd video images of the gel were grabbed into a Macintosh microcomputer using a Scion grabber card, and band intensities were analysed using NIH Image (<http://rsb.info.nih.gov/nih-image/>). The concentration of motor in pellet and supernatant was plotted using Kaleidagraph 3.05 (Synergy software), and binding parameters were obtained by fitting rectangular hyperbolae by least squares analysis.

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 μ l 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. 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 μ M Nkin460GST and 0.225 μ M Nkin360 were used as final concentrations in the assay. For the measurement of basal ATPase 5 μ M motor was used.

Stopped flow analysis

Rapid mixing experiments were performed with a Hitech SFX61 spectrofluorimeter (HiTech Scientific, Salisbury, UK). MT concentrations were saturating for the ATPase, based on steady-state assays of MT-activated ATPase. The final concentrations were 0.5 μ M motor, 2 μ M mantATP, 20 and 30 μ M 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, ATPyS, ADP). In addition, 25 mM NaCl was added for experiments with Nkin460GST. Nkin460GST or Nkin360 were labelled with mantADP by incubating 1 μ M motor with 4 μ M mantATP at 21°C for 15 min. To observe the decrease of fluorescence corresponding to MT-activated release of mantADP from the active sites, 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 with MT for 5 min 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 Axiovert 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 Isle Optics, UK) in the arrangement described by Molloy *et al.* (1995). The objective lens was the Zeiss 100 $\times$, 1.25NA oil immersion. A standard Zeiss 100 W tungsten/halogen lamp house was used for illumination. A custom-made condenser (using 40 $\times$, 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 adjustment was carried out using a feedback-controlled piezoelectric XY stage (50 $\times$ 50 μ m 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, S-1557-03) allowing the bead position to be measured in two axes (XY) with sub-nanometre accuracy. The detector circuitry 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.

Trap calibrations

A quadrant calibration and a trap stiffness calibration were carried out for each trapped bead. The quadrant detector was calibrated by steering the trap a known distance in both axes. Trap stiffness (X and Y) was calculated by the average of three methods: a Stokes' drag calibration, a mean-square bead displacement calibration, and by fitting a Lorentzian curve to the Power spectrum of the Brownian movement of the bead (Visscher and

Block, 1998). In the Stokes and Fourier calibrations, proximity to the coverslip is corrected for, as described by Svoboda and Block (1994). These calibrations are controlled in the software and take ~1 min to complete. If any of the three stiffness calibrations varied by >20% from the mean calculated value, that bead was rejected. Such variation may occur for several reasons: a non-spherical bead, bead radius estimation error, proximity of another free bead or a MT in solution near to (or stuck to) the trapped bead.

MT gliding assay flow cell preparation

All solutions used in the gliding assays were based on the BRB 80 solution (described previously) supplemented with 5 mM DTT and 10 μ M taxol ('BRB-supp'). In assays where the ATP concentration was below 100 μ M, an ATP regenerating system was used (50 μ g/ml creatine phosphokinase and 1 mM phosphocreatine). Flow cells were made using two ethanol-washed coverslips (50 $\times$ 22 and 22 $\times$ 22 mm, BDH) sealed using silicone 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 μ M, depending on motor density required) along with 100 μ g/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 μ l. 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.mcrl.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 μ M taxol and 2 mM phosphocreatine. Polystyrene beads (0.75 μ m 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 μ g/ml casein). The Nkin-His motor was added at a concentration of 1 nM or less (beads are ~0.02 nM 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 μ g/ml glucose oxidase and 20 μ g/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 directly supported coverslip in this way removes the problem of focus drift due to movement of the flow cell sealant.

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