ADP release is the rate-limiting step of the MT activated ATPase of non-claret disjunctional and kinesin

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Received 17 May 1995

Abstract **The motor protein non-claret disjunctional (ncd) moves towards the minus ends of microtubules (MTs), whereas its close relative kinesin moves in the opposite direction towards the plus ends of MTs. The mechanisms of movement and directional reversal for these motor proteins are unknown. Here we report the rate constants for MT activated ADP release from a recombinant double-headed ncd protein, GST-MC5, and a recombinant double-headed kinesin protein, KΔ401, measured using the fluorescent nucleotide analogues methylanthranilyol ATP (mantATP) and mantADP. Comparison of the maximal MT activated mantADP release rates for these proteins with their maximal MT activated mantATP turnover rates indicates that ADP release is the rate-limiting step for ATP turnover for both ncd and kinesin. This data supports the view that directional reversal may result from structural rather than chemical kinetic differences in the way the motors interact with MTs.**

Key words: ncd; Kinesin; Molecular motor; Microtubule motor

1. Introduction

Molecular motors are able to convert chemical energy, derived from ATP hydrolysis, into directed movement along either actin or microtubule (MT) tracks. Understanding their kinetic mechanism is thus fundamental to understanding their mechanism of motion [1]. Kinetics examines when attachment and detachment occur, which nucleotide intermediates predominate and in the case of the oppositely directed MT motors, kinesin and non claret disjunctional (ncd), offers possibilities for exploring the sources of directionality [2,3].

Kinesin superfamily motors move their cargoes in a defined direction along microtubules (MTs) using ATP as their fuel. Different members of the superfamily move towards either the plus end or the minus end of MTs [4]. The direction of movement is believed to an intrinsic property of the motor domain (containing the ATPase activity and MT binding properties of the motor) which is highly conserved across the superfamily [5]. The mechanisms of movement and direction reversal are unknown, although two models to explain directionality have been discussed. The first possible model proposes that by altering the mechanochemical coupling of one motor relative to the other, force would be generated in the opposite direction [6]. In the second model the motors have similar chemistry and chemomechanical coupling, and directional reversal results from a structural about face within the track–motor complex [7].

Several studies have investigated the transient and steady-state kinetics of kinesin [1,8–11], although to date only steady-state kinetics on ncd have been reported [7]. In the absence of MTs the basal ATPase activity of both motors is slow and ADP release is rate limiting [7,12]. Interaction with MTs stimulates the ATPase activity of both motors at least 1000-fold ensuring that ATP turnover and the conformational changes associated with force generation are intimately coupled [13]. There is, however, some uncertainty over which step of kinesin's MT activated ATPase cycle is rate limiting.

Recent work suggests two different candidates for the rate-limiting step. Hackney originally proposed that ADP release was rate-limiting in the presence of MTs [12] and a more extensive study by Taylor reached the same conclusions [14,15]. Gilbert and Johnson also proposed a mechanism in which ADP release was rate limiting [16]. These workers now, however, believe that an earlier step in the cycle must be rate limiting as they found ADP release rates ~10-fold faster than the steady-state rate of ATP hydrolysis [1]. Their data suggested that the dissociation of kinesin from the MT after ATP hydrolysis was probably the rate-limiting step.

The issue of which step is rate limiting in the activated ATPase is important. Force generation requires a coupling of the hydrolytic cycle of the motor to its force generating conformational changes [13], so that the rates of chemical steps report the rates of associated conformational (mechanical) steps. Slow steps in the MT activated ATPase pathway identify which of the conformational states along the path have long lifetimes. Long-lived strong binding states are likely to be associated with force generation and the power stroke [1], whilst long-lived weak binding states indicate an enrichment of motor in a detached or detachable conformation. For both kinesin and ncd, the transition from weak to strong binding appears to be coupled to ADP release [7,15,17]. Here we show that this ADP release step is rate limiting for both motors.

2. Materials and methods

2.1. Reagents

General laboratory reagents were purchased from Sigma (Poole, Dorset, UK). *N*-Methylisatoic anhydride used for the synthesis of mant nucleotides was obtained from Molecular Probes Inc. (Cambridge Bioscience, Cambridge, UK).

2.2. Expression and purification of GST-MC5 and KΔ401

The construction of the vectors for the expression of GST-MC5 and KΔ401 and protocols for their purification have been described previously [18,19]. The concentrations of GST-MC5 and KΔ401 are expressed per single chain.

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Abbreviations: MTs, microtubules; ncd, non claret disjunctional; mant, methylanthranilyol; GST, glutathione *S*-transferase.

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SSDI 0014-5793(95)00723-7

2.3. Summary of physical and kinetic properties of GST-MC5 and KΔ401

GST-MC5 contains residues 295–700 of ncd fused to the C-terminus glutathione *S*-transferase (GST). The fusion protein forms a mixture of dimers and tetramers in solution [18] although we have found that this does not appear to alter its kinetic behaviour [7,19]. KΔ401 contains residues 1–401 of the rat kinesin heavy chain and forms stable dimers in solution [19]. Both motors possess low ATPase activities in the absence of MTs with ADP release the rate-limiting step. The rates of ADP release are 0.0036 s^{-1} for GST-MC5 and 0.005 s^{-1} for KΔ401 in the absence of MTs [19]. GST-MC5 and KΔ401 have strong MT activated ATPases with k_{cat} values of 1.1 s^{-1} and 21 s^{-1}, respectively [7,19]. The binding of both motors to MTs has also been well characterised and at saturation the stoichiometries of binding for both constructs to MTs are one motor molecule per tubulin heterodimer [7,19].

2.4. Purification and polymerisation of tubulin

Tubulin was purified from porcine brain and polymerised to form MTs as described [19]. Following polymerisation MTs were stabilised with 20 μM taxol, pelleted (100000 × *g*, 20 min, 25°C) and resuspended in assay buffer in order to remove guanine nucleotides. MT concentrations are expressed per tubulin monomer.

2.5. Synthesis and purification of mant nucleotides

mantATP and mantADP were synthesised as described [20] and purified on a DEAE cellulose column with a 0.1 M to 1 M triethylamine (pH 7.5) gradient.

2.6. Measurement of MT activated rates of mantADP release from GST-MC5 and KΔ401

All experiments were performed in 20 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA and 20 μM taxol at 20°C. GST-MC5 (0.2 μM) and KΔ401 (0.5 μM) were incubated with a 4-fold excess of either mantATP or mantADP until the exchange reaction was complete (typically 20 min at 20°C). The fluorescent motor–mantADP complexes were placed in one syringe and MTs plus 2 mM ATP in the second syringe of a Hi-Tech SF-61MX stopped flow spectrofluorimeter (Hi-Tech Scientific Ltd., Salisbury). The two reactants were rapidly mixed and the release of mantADP monitored using the fluorescence channel.

2.7. Measurement of steady-state MT activated turnover rates using mantATP as a substrate

The steady-state MT activated turnover of mantATP by GST-MC5 and KΔ401 was quantified using a discontinuous malachite green assay which detects the production of inorganic phosphate [21]. Assays were performed at 20°C in 20 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA, 2 mM mantATP and 20 μM taxol.

2.8. Measurement of MT activated rates of mantADP release from KΔ401 using conditions described by Gilbert et al. [1]

Stopped flow experiments were performed at 25°C in 20 mM HEPES, pH 7.2, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate and 1 mM DTT. Motor–mantADP complexes were formed by incubating KΔ401 (4 μM) with mantADP (4 μM) for 20 min at 25°C. The fluorescent complexes were placed in one syringe and MTs (10 μM or 40 μM) plus 2 mM ATP were placed in the second syringe of the stopped flow spectrofluorimeter. The two reactants were rapidly mixed and the release of mantADP monitored using the fluorescence channel.

3. Results

3.1. Measurement of ADP release from GST-MC5

Two well-characterised recombinant proteins were used in this study, a double-headed ncd protein, GST-MC5, and a double-headed kinesin protein, KΔ401 (Fig. 1). The physical and kinetic properties of these motors are summarised in section 2.3. The rates of ADP release from these motors were measured using the fluorescent nucleotide analogues methylanthranilyol-ATP (mantATP) and mantADP. These nucleotide analogues have been widely employed in kinetic studies of kinesin [1,11,15], ncd [7] and myosin [20,22].

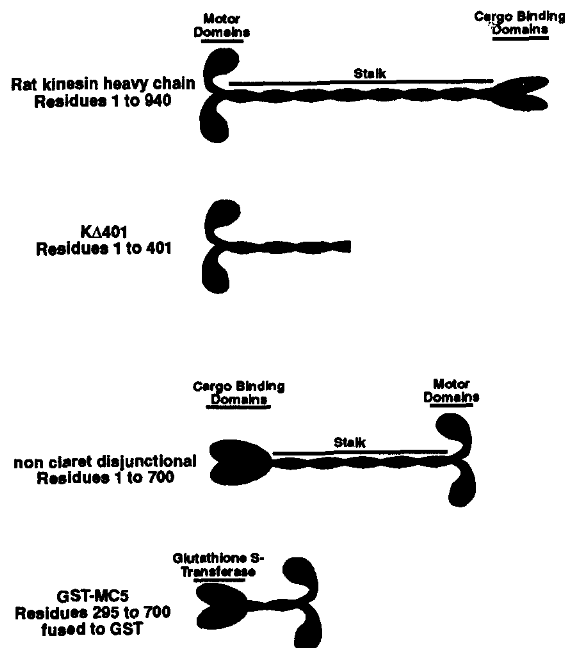
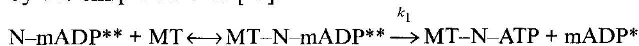


Fig. 1. Schematic representation of the recombinant proteins GST-MC5 and KΔ401. GST-MC5 was formed by the fusion of residues 295–700, containing all of the motor domain and part of the coiled-coil region, to the C-terminus of glutathione *S*-transferase. KΔ401 consists of residues 1–401 of the rat kinesin heavy chain clone and contains all of the motor domain and part of the stalk region.

Both GST-MC5 and KΔ401 are purified with stoichiometric amounts of ADP in their active sites [7,19]. Incubating the motors with either mantATP or mantADP results in the exchange of the mant nucleotide with the ADP in the active site. The fluorescence of the mant nucleotide is enhanced upon binding resulting in the formation of fluorescent motor.mantADP complexes.

The fluorescent complexes were rapidly mixed with MTs over a range of concentrations in the presence of 1 mM ATP (to stop any rebinding of the mantADP) in a stopped flow spectrofluorimeter. The reaction monitored in the stopped flow comprises a binding step followed by a fluorescence decay as the mantADP is released from the motor. It can be modelled by the simple scheme [15]:



$$\text{N–mADP}^{**} + \text{MT} \longleftrightarrow \text{MT–N–mADP}^{**} \xrightarrow{k_1} \text{MT–N–ATP} + \text{mADP}^{*}$$

where N–mADP** is the fluorescent GST-MC5–mantADP complex. The absence of a lag phase in the fluorescence traces (Fig. 2a) indicates that the initial binding is a rapid equilibrium step and that k_1 is the step that determines the rate of mantADP release under these conditions.

The rates of mantADP release from GST-MC5 were MT concentration dependent and the data were fitted to a rectangular hyperbola (Fig. 2b). The maximal rate of mantADP release extrapolated from this fit was 1.56 (±0.14) s^{-1}. The concentration of MTs required for half-maximal rate ($K_{50\%,\text{mADP}}$) is a measure of the dissociation constant of the N–mADP** complex and was 1.35 (±0.60) μM. These values were identical (within experimental error) irrespective of whether the

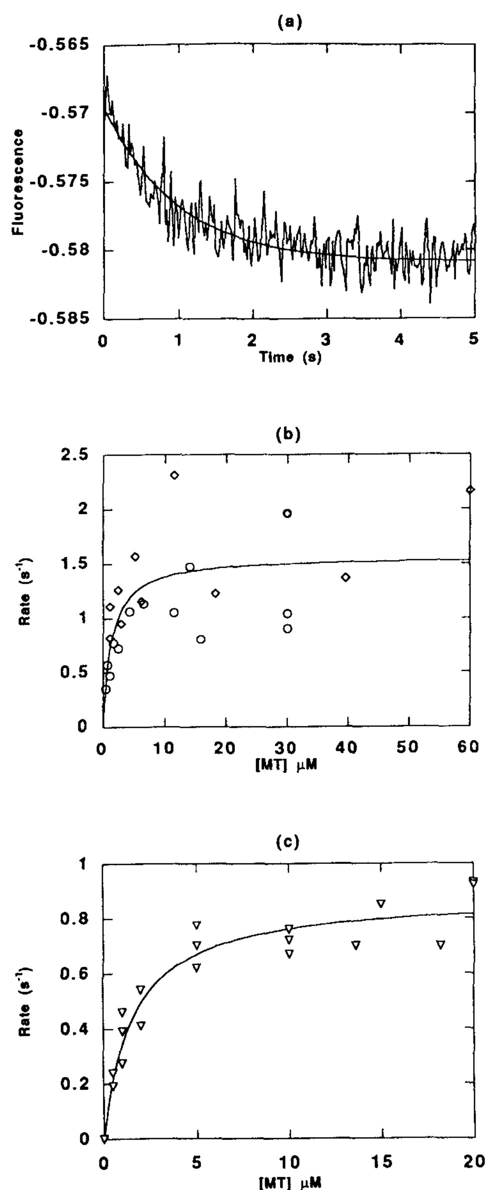


Fig. 2. (a) Representative stopped flow trace for GST-MC5 (0.1 μM) labelled with mantADP (0.4 μM) rapidly mixed with MTs (30 μM) and ATP (1 mM). All concentrations are those in the flow cell after mixing. The solid black line represents the best fit of the data to a single exponential and gives a mantADP release rate of 1.06 s^{-1}. (b) MT induced rates of mantADP release from GST-MC5 preincubated with either (○) mantADP or (◇) mantATP. Each data point is the average of typically three separate traces. The solid black line shows the best fit of the data to a rectangular hyperbola and gives a maximal rate of ADP release of 1.56 (±0.14) s^{-1} and a $K_{50\%,mADP}$ of 1.35 (±0.60) μM. (c) Steady-state measurement of MT activated turnover of mantATP by GST-MC5. The data were fitted to a rectangular hyperbola and give a maximal mantATP hydrolysis rate of 0.88 (±0.04) s^{-1} and a $K_{50\%,SSmATP}$ of 1.58 (±0.30) μM.

mantADP at the nucleotide binding site was loaded via mantATP hydrolysis or via direct exchange of mantADP (Fig. 2b).

3.2. Measurement of ADP release from KΔ401

The MT dependency of mantADP release from KΔ401 was measured using the same method. Fluorescent KΔ401 mantADP complexes were rapidly mixed with MTs over a range of concentrations in the presence of 1 mM ATP. No lag phase was observed upon mixing the KΔ401–mantADP complexes with MTs (Fig. 3a) indicating that the reaction could be treated in the same manner as that of GST-MC5. The rates of mantADP release increased with a hyperbolic dependence on MT concentration (Fig. 3b) to give a maximal rate of mantADP

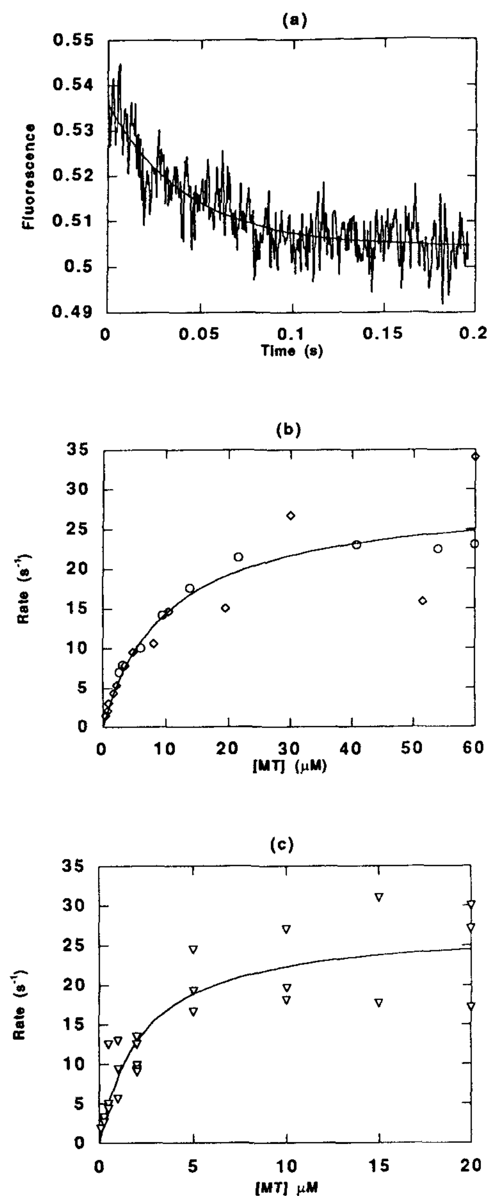


Fig. 3. (a) Representative stopped flow trace for KΔ401 (0.25 μM) labelled with mantADP (1 μM) rapidly mixed with MTs (60 μM) and ATP (1 mM). All concentrations are those in the flow cell after mixing. The solid black line represents the best fit of the data to a single exponential and gives a mantADP release rate of 22.93 s^{-1}. (b) MT induced rates of mantADP release from KΔ401 preincubated with either (○) mantADP or (◇) mantATP. Each data point is the average of typically three separate traces. The solid black line shows the best fit of the data to a rectangular hyperbola and gives a maximal rate of ADP release of 29.04 (±2.51) s^{-1} and a $K_{50\%,mADP}$ of 10.29 (±2.66) μM. (c) Steady-state measurement of MT activated turnover of mantATP by KΔ401. The data were fitted to a rectangular hyperbola and give a maximal mantATP hydrolysis rate of 27.32 (±2.31) s^{-1} and a $K_{50\%,SSmATP}$ of 2.28 (±0.64) μM.

Table 1
Summary of the kinetic parameters for KΔ401 and GST-MC5

	KΔ401	GST-MC5
$k_{cat,mADP}$ (s^{-1})	29.03 (±2.51)	1.56 (±0.14)
$K_{50\%,mADP}$ (μM)	10.29 (±2.66)	1.35 (±0.60)
$k_{cat,SSmATP}$ (s^{-1})	27.32 (±2.31)	0.88 (±0.04)
$K_{50\%,SSmATP}$ (μM)	2.28 (±0.64)	1.58 (±0.30)

The maximal rates of mantADP release ($k_{cat,mADP}$) and the amounts of MTs required to give half-maximal rates of release ($K_{50\%,mADP}$) were derived from best fits to the data in Fig. 2b and 3b. The maximal steady-state rates of mantATP hydrolysis ($k_{cat,SSmATP}$) and the amounts of MTs required to give half-maximal velocity ($K_{50\%,SSmATP}$) were derived from the best fits to the data in Fig. 2c and 3c. All $K_{50\%}$ values are expressed per tubulin monomer concentration.

release of 29.03 (±2.51) s^{-1} and a $K_{50\%,mADP}$ of 10.29 (±2.66) μM. As for GST-MC5, the rates of mantADP release from KΔ401 were identical irrespective of whether the protein had been preincubated with mantATP or mantADP.

3.3. *MT activated steady-state rates of mantATP turnover by GST-MC5 and KΔ401*

MT activated steady-state measurements were performed for GST-MC5 and KΔ401 using mantATP as the substrate. The maximal rates of hydrolysis (k_{cat}) under these conditions were 0.88 (±0.04) s^{-1} for GST-MC5 (Fig. 2c) and 27.32 (±2.31) s^{-1} for KΔ401 (Fig. 3c). These rates compare with the values of 1.10 (±0.06) s^{-1} for GST-MC5 [7] and 20.83 (±0.83) s^{-1} for KΔ401 [19] using ATP as the substrate.

The amounts of MTs required for half-maximal activation ($K_{50\%,SSmATP}$) were 1.58 (±0.30) μM for GST-MC5 and 2.28 (±0.64) μM for KΔ401. These compare with values of 3.02 (±0.49) μM for GST-MC5 [7] and 0.79 (±0.14) μM for KΔ401 [19] using ATP as the substrate.

The similarity in the k_{cat} and $K_{50\%}$ values obtained using mantATP and ATP indicate that the mantATP is a reasonable analogue of ATP and that the mantADP release rates are good estimates of the ADP release rate.

4. Discussion

ATP turnover by ncd is a multistep process and as a consequence each step in the pathway must be faster than the steady-state turnover rate [15,23]. The maximal rate of mantADP release from GST-MC5 was found to be approximately twice that of its maximal turnover rate. This result is consistent with the ADP release step making a dominant contribution to the overall turnover rate of ncd. Assuming a maximum cycle time of ~1000 ms for GST-MC5 (k_{cat} of ~1 s^{-1}) these results indicate that the motor spends at least 600 ms (~60% of its cycle time) in the ADP state.

No detailed data has been published concerning the earlier steps in the kinetic cycle of ncd except that phosphate (P_i) release from the motor, in the absence of MTs, occurs much faster ($\gg$0.1 s^{-1}) than ADP release [7]. There are at least three steps prior to ADP release from the motor, ATP binding, ATP hydrolysis and P_i release. These must occur during the remainder of the cycle time (which is ~400 ms) making it unlikely that there is another comparably slow step in the cycle. The data therefore indicate that the motor. ADP complex is the predominant nucleotide state of ncd during MT activated ATP turnover and that ADP release from the motor is the rate-limiting step of the ATPase cycle.

The mantADP chase experiments performed with KΔ401 also indicate that the ADP release step makes a major contribution to the turnover rate of kinesin. The small difference between the maximal mantADP release rate and the steady-state turnover rate suggests, that compared to ncd, the motor may be spending a greater proportion of its cycle time in the ADP state. The results, however, suggest the same general conclusion, namely, that ADP release is the rate-limiting step of the MT activated ATPase of kinesin. Previously we had demonstrated that the steady-state kinetics of ncd and kinesin were similar in so far as ADP release in the absence of MTs was rate limiting [7]. The current data provides evidence that the ADP step release is also rate limiting for both motors in the presence of MTs.

It should be noted, however, that the maximum ADP release rate from KΔ401 is markedly different from that reported by Gilbert et al. [1] who used the same sized construct (called K401), but derived from a *Drosophila* kinesin clone. They reported maximal rates of mantADP release of ~300 s^{-1}. This was in contrast to maximal steady-state ATPase rates for K401 of 20 s^{-1}. Their data suggests that for the MT activated ATPase ADP release is no longer the rate-limiting step of the cycle and that it must occur earlier in the ATPase cycle. The magnitude of the difference in the mantADP release rates cannot be accounted for by the different conditions used in the two sets of experiments. We repeated our experiments at two MT concentrations under the conditions reported by Gilbert et al. (see section 2.7) and found rates about ten-fold lower than they reported. At 5 μM and 20 μM MTs we found rates of mantADP release of 6.73 s^{-1} and 17.54 s^{-1}, respectively. These compare with rates of ~90 s^{-1} and ~170 s^{-1} reported at these MT concentrations by Gilbert et al. [1].

These large differences in the ADP off rates cannot easily be accounted for at present. Our steady-state data are in good agreement with Gilbert et al., but we find a ten-fold slower rate of MT activated ADP release in the chase experiments. One possibility we cannot test at present is that this is due to the use of recombinant kinesin from different sources (rat and *Drosophila*). A recent study, however, using a human kinesin construct (called K379) reports rates of mantADP release comparable to those measured in the present study [14,15].

Comparison of the MT dependence of mantADP release ($K_{50\%,mADP}$) and mantATP hydrolysis ($K_{50\%,SSmATP}$) indicates a clear and significant difference between the GST-MC5 and KΔ401. The ratio of $K_{50\%,mADP}/K_{50\%,SSmATP}$ values is ~1 for GST-MC5 and ~4 for KΔ401 (Table 1). The kinetic constant $K_{50\%,SSmATP}$, which demonstrates the dependence of ATP turnover on MT concentration, by itself provides little information as it is determined by all of the steps along the reaction pathway [24]. The mantADP release data provides information on a defined part of the pathway with the $K_{50\%,mADP}$ a measure of the dissociation constant of the motor.mantADP complex.

The similarity of the $K_{50\%,mADP}$ and the $K_{50\%,SSmATP}$ values for GST-MC5 is consistent with the interaction of the GST-MC5.ADP state with the MTs making the most significant contribution to the MT dependence of the steady-state cycling rate of ncd. This is in contrast to KΔ401. The four-fold difference in the $K_{50\%,mADP}$ and the $K_{50\%,SSmATP}$ values indicates that

the interaction of other motor–nucleotide states with MTs contribute to the MT dependence of the steady-state cycling rate of kinesin.

The finding that the same chemical step is rate limiting for the MT activated turnover of ATP by these oppositely directed motors provides further evidence that kinesin and ncd have similar chemomechanical cycles [7]. This supports the view that directional reversal may result from structural rather than kinetic differences in the way the motors interact with the MTs [7]. However, there is evidence from our data that the pathways by which the two motors interact with MTs during ATP turnover may be subtly different. Although, this would be predicted by a change in the coupling between the two motors [6] we feel that on balance the evidence suggests that directional reversal results from a structural differences in the way the two motors interact with MTs. Further studies investigating the binding of ncd and kinesin to MTs under different nucleotide states will be necessary to address this issue.

Acknowledgements: We thank Dr. Sharyn Endow for the kind gift of the GST-MC5 clone and Drs. Scott Brady and Anne Sperry for the kind gift of the rat kinesin heavy chain clone. We are also grateful to Dr. Isabelle Crevel for the kind gift of the tubulin used in these experiments and the BBSRC for the kind gift of the Hi-Tech stopped flow spectrofluorimeter.

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