A Mechanistic Model for Ncd Directionality*

Received for publication, September 12, 2000, and in revised form, January 19, 2001
Published, JBC Papers in Press, March 2, 2001, DOI 10.1074/jbc.M008347200

Kelly A. Foster‡, Andrew T. Mackey, and Susan P. Gilbert§
From the Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Ncd is a kinesin-related protein that drives movement to the minus-end of microtubules. Pre-steady-state kinetic experiments have been employed to investigate the cooperative interactions between the motor domains of the Ncd dimer and to establish the ATPase mechanism. Our results indicate that the active sites of dimeric Ncd free in solution are not equivalent; ADP is held more tightly at one site than at the other. Upon microtubule binding, fast release of ADP from the first motor domain is stimulated at 18 s⁻¹, yet rate-limiting ADP release from the second motor domain occurs at 1.4 s⁻¹. We propose that the head with the low affinity for ADP binds the microtubule first to establish the directional bias of the microtubule-Ncd intermediate where one motor domain is bound to the microtubule with the second head detached and directed toward the minus-end of the microtubule. The force generating cycle is initiated as ATP binds to the empty site of the microtubule-bound head. ATP hydrolysis at head 1 is required for head 2 to bind to the microtubule. The kinetics indicate that two ATP molecules are required for a single step and force generation for minus-end directed movement generated by this non-processive dimeric motor.

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

* This work was supported in part by National Institutes of Health Grant GM 54141 (to S. P. G.), American Cancer Society Grant IRG-58-35, and in part by a Basil O’Connor Starter Scholar Research Award 5-FY95–1136 from the March of Dimes Birth Defects Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ University of Pittsburgh Chancellor’s Scholar and the recipient of a Beckman Scholarship from the Arnold and Mabel Beckman Foundation and a Barry M. Goldwater Scholarship in support of undergraduate research. Present address: Dept. of Neuroscience, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115.
§ Recipient of American Cancer Society Junior Faculty Research Award JFRA-618. To whom correspondence should be addressed: Dept. of Biological Sciences, 518 Langley Hall, University of Pittsburgh, Pittsburgh, PA 15260. Tel.: 412-624-5842; Fax: 412-624-4759; E-mail: spg1+@pitt.edu

‡ The abbreviations used are: Ncd, non-claret disjunctional; MC1, Ncd construct consisting of amino acid residues Leu99–Lys700; mant-ADP, 2′(3′)-O-(N-methylanthraniloyladenosine 5′-diphosphate; mant-ATP, 2′(3′)-O-(N-methylanthraniloyladenosine 5′-triphosphate; ATP-γS, adenosine 5′-(thiotriphosphate); AMP-PNP, 5′-adenylly-β,γ-imidodiphosphate; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate.

The Journal of Biological Chemistry Vol. 276, No. 22, Issue of June 1, pp. 19259–19266, 2001
Printed in U.S.A.
filtration and stopped-flow kinetics reveal that the two heads within the dimer are different in solution in the absence of microtubules, with one head binding ADP weakly and one head binding ADP tightly. Stopped-flow experiments indicate that the two heads of the dimer release ADP at different rates after interacting with the microtubule. We propose that the head with the low affinity for ADP binds the microtubule first to establish the intermediate captured by cryo-EM with the detached head directed toward the minus-end of the microtubule. The kinetics reveal cooperative interactions within the dimer that account for the orientation of this directionally biased Mt-Ncd intermediate. The kinetics also establish a distinctive ATPase mechanism for Ncd and provide insight into the mechanochemistry variability among kinesin superfamily members.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]ATP (>3,000 Ci/mmol) was purchased from PerkinElmer Life Sciences, polyethyleneimine-cellulose F TLC plates (EM Science of Merck, 20 × 20 cm, plastic backed) from VWR Scientific (West Chester, PA), and taxol (Taxis brevifolia) from Calbiochem. ATP, GTP, DEAE-Sepharose FF, and SP-Sepharose were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The N-methylthraniloyl derivatives of adenine nucleotides (mant-ATP and mant-ADP) were synthesized and characterized as described previously (15, 28, 35).

**Buffer Conditions**—The following buffer was used for the experiments described: ATPase buffer at 25 °C (20 mM HEPES, pH 7.2, with KOH, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM Mg acetate, 50 mM K acetate, 1 mM dithiothreitol, 5% sucrose). Protein Purification—The dimeric Ncd construct (MC1) was expressed in the Escherichia coli cell line BL21(DE3) from a clone generously provided by Dr. Sharyn Endow, Duke University Medical Center (36). This MC1 construct was expressed as a nonfuson protein and contains amino acid residues Leu299-Phe614; therefore, the N-terminal ATP-independent microtubule-binding site is absent. MC1 was purified and DEAE-Sepharose FF and SP-Sepharose were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The N-methylthraniloyl derivatives of adenine nucleotides (mant-ATP and mant-ADP) were synthesized and characterized as described previously (15, 28, 35).

**Pre-steady-state Experiments**—The following buffer was used for the experiments described: ATPase buffer at 25 °C (20 mM HEPES, pH 7.2, with KOH, 0.1 mM EDTA, 0.1 mM EGTA, 5 mM Mg acetate, 50 mM K acetate, 1 mM dithiothreitol, 5% sucrose).

**Protein Purification**—The dimeric Ncd construct (MC1) was expressed in the Escherichia coli cell line BL21(DE3) from a clone generously provided by Dr. Sharyn Endow, Duke University Medical Center (36). This MC1 construct was expressed as a nonfusion protein and contains amino acid residues Leu299-Phe614; therefore, the N-terminal ATP-independent microtubule-binding site is absent. MC1 was purified and characterized as described previously (15, 28, 35). Four different MC1 preparations were used for the pre-steady-state experiments reported, and the steady-state parameters were comparable to those reported previously: $k_{in} = 2 \times 10^{-2}$, $K_{d,ATP} = 23 \mu M$, and $K_{d,ADP} = 20 \mu M$. MC1 is dimeric under the conditions of the experiments reported here based on the $K_{d}$ for dimerization for MC1 at $<5$ nM (33). For the pre-steady-state experiments reported, we did not pretreat the Mt-Ncd complex with apyrase to remove bound ADP. This approach was chosen due to concern that the Ncd protein behavior may be altered by the treatment, leading to a significant fraction of inactive protein associating with the Mt. All concentrations subsequently provided by Dr. Sharyn Endow, Duke University Medical Center (36). This MC1 construct was expressed as a nonfusion protein and contains amino acid residues Leu299-Phe614; therefore, the N-terminal ATP-independent microtubule-binding site is absent. MC1 was purified and characterized as described previously (15, 28, 35). Four different MC1 preparations were used for the pre-steady-state experiments reported, and the steady-state parameters were comparable to those reported previously: $k_{in} = 2 \times 10^{-2}$, $K_{d,ATP} = 23 \mu M$, and $K_{d,ADP} = 20 \mu M$. MC1 is dimeric under the conditions of the experiments reported here based on the $K_{d}$ for dimerization for MC1 at $<5$ nM (33). For the pre-steady-state experiments reported, we did not pretreat the Mt-Ncd complex with apyrase to remove bound ADP. This approach was chosen due to concern that the Ncd protein behavior may be altered by the treatment, leading to a significant fraction of inactive protein associating with the Mt. All concentrations were synthesized and characterized as described previously (15, 28, 35).

**Acid Quench Experiments**—The rate constant of ATP hydrolysis was measured using a rapid chemical quench-flow instrument (KinTek Corp.) at 25 °C in ATPase buffer. ATP hydrolysis was measured by rapidly mixing the preformed Mt-N complex (1 μM MC1, 20 μM tubulin, 20 μM taxol, final after mixing) with increasing concentrations of ATP. The stopped-flow transients in Fig. 3 were each fit to a single exponential function plus a linear term to obtain the rate and the amplitude of the observed process of mant-ADP release. The amplitudes were fit to the biphasic burst equation, $A(t) = A_{max} \times [1 - \exp(-k_{is}t)] + k_{is}N_{0}$. ATP. The fit of the data to a hyperbola provided the $K_{d,ATP}$ in the presence of either 50 μM ATP or 1 mM AMP-PNP (Fig. 3D). The apparent $K_{d,ATP,0}$ and $K_{d,ATP,P}$ were obtained from Equation 1.

**Acid Quench Experiments**—The rate constant of ATP hydrolysis was measured using a rapid chemical quench-flow instrument (KinTek Corp.) at 25 °C in ATPase buffer. ATP hydrolysis was measured by rapidly mixing the preformed Mt-N complex (1 μM MC1, 20 μM tubulin, 20 μM taxol, final after mixing) with increasing concentrations of ATP. The stopped-flow transients in Fig. 3 were each fit to a single exponential function plus a linear term to obtain the rate and the amplitude of the observed process of mant-ADP release. The amplitudes were fit to the biphasic burst equation, $A(t) = A_{max} \times [1 - \exp(-k_{is}t)] + k_{is}N_{0}$. ATP. The fit of the data to a hyperbola provided the $K_{d,ATP}$ in the presence of either 50 μM ATP or 1 mM AMP-PNP (Fig. 3D). The apparent $K_{d,ATP,0}$ and $K_{d,ATP,P}$ were obtained from Equation 1.

**Acid Quench Experiments**—The rate constant of ATP hydrolysis was measured using a rapid chemical quench-flow instrument (KinTek Corp.) at 25 °C in ATPase buffer. ATP hydrolysis was measured by rapidly mixing the preformed Mt-N complex (1 μM MC1, 20 μM tubulin, 20 μM taxol, final after mixing) with increasing concentrations of ATP. The stopped-flow transients in Fig. 3 were each fit to a single exponential function plus a linear term to obtain the rate and the amplitude of the observed process of mant-ADP release. The amplitudes were fit to the biphasic burst equation, $A(t) = A_{max} \times [1 - \exp(-k_{is}t)] + k_{is}N_{0}$. ATP. The fit of the data to a hyperbola provided the $K_{d,ATP}$ in the presence of either 50 μM ATP or 1 mM AMP-PNP (Fig. 3D). The apparent $K_{d,ATP,0}$ and $K_{d,ATP,P}$ were obtained from Equation 1.

**Acid Quench Experiments**—The rate constant of ATP hydrolysis was measured using a rapid chemical quench-flow instrument (KinTek Corp.) at 25 °C in ATPase buffer. ATP hydrolysis was measured by rapidly mixing the preformed Mt-N complex (1 μM MC1, 20 μM tubulin, 20 μM taxol, final after mixing) with increasing concentrations of ATP. The stopped-flow transients in Fig. 3 were each fit to a single exponential function plus a linear term to obtain the rate and the amplitude of the observed process of mant-ADP release. The amplitudes were fit to the biphasic burst equation, $A(t) = A_{max} \times [1 - \exp(-k_{is}t)] + k_{is}N_{0}$. ATP. The fit of the data to a hyperbola provided the $K_{d,ATP}$ in the presence of either 50 μM ATP or 1 mM AMP-PNP (Fig. 3D). The apparent $K_{d,ATP,0}$ and $K_{d,ATP,P}$ were obtained from Equation 1.
to the pre-washed centrifuge column (performed in duplicate) and centrifuged at \(-1000 \times g\) for 5 min at 22 °C in a bench-top swinging bucket centrifuge (2450 rpm, Sorvall RT 6000B Refrigerated Tabletop Centrifuge). Approximately 80 μl (79–81.5 μl) was recovered as the void volume for analysis by the Bradford Assay for protein concentration and liquid scintillation counting for nucleotide concentration determination. Aliquots of 5, 7, and 10 μl were used to determine total counts for the calculation of nucleotide concentration, and aliquots of 10 and 15 μl were used to determine protein concentration. Parallel experiments were included as controls in which either no protein was used in the reaction or dimeric kinesin K401 or ovalbumin was used. These control experiments assessed the degree of nonspecific binding of the MC1 to the gel filtration resin, whether all free nucleotide partitioned within the bead pores, and whether there were other inconsistencies in the assay procedure. Kinetic modeling and simulations were performed using Scheme 1 with Scientist software (MicroMath Scientific Software, Salt Lake City, UT).

RESULTS

**ADP Release from Head 2**—The rate of mant-ADP release from the Mt-MC1 complex was measured previously by rapidly mixing the preformed MC1-mant-ADP complex with microtubules in the presence of MgATP in the stopped-flow (15). A maximum observed rate of 3.7 s\(^{-1}\) was obtained, and the kinetics represent mant-ADP dissociation from both motor domains. We then pursued experiments to measure directly the kinetics of mant-ADP release from each motor domain of the Ncd dimer. Cryo-EM studies have revealed a stable Mt-ADP intermediate in which one motor domain is bound to the microtubule, yet the second motor domain is detached and directed toward the minus-end of the microtubule (17–21). For our experiments, we assumed that the detached motor domain would bind mant-ADP more tightly than the motor domain bound to the microtubule (experimental design shown in Fig. 1B) because microtubules activate ADP release from 0.005 s\(^{-1}\) in the absence of microtubules to \(-2 \text{ s}^{-1}\) at high microtubule concentrations (14, 15, 30–34, 37). To determine the rate of mant-ADP release from the detached motor domain (head 2), microtubules, MC1, and mant-ADP were preincubated to form the Mt-MC1-mant-ADP complex (1 mant-ADP per MC1 dimer). This complex was rapidly mixed with MgATP to initiate mant-ADP release from head 2.

Fig. 1 shows the time dependence of the fluorescence change as mant-ADP is released from the more hydrophobic active site of the motor domain to the solution where the fluorescence is quenched. Both the rate and the amplitude associated with the fluorescence exponential phase increases as a function of ATP concentration. The rate of mant-ADP release from head 2 is ATP concentration dependent with the maximum rate constant at 1.4 s\(^{-1}\) (K\(_{1/2}\) = 0.6 μM ATP). These results suggest that ADP release from head 2 is rate-limiting for steady-state turnover because all other steps in the pathway have been determined to be significantly faster than the k\(_{a}\) at 2 s\(^{-1}\) (14, 15). This experiment also implies that ATP binding at the first head is necessary for the second head to bind the microtubule and release its mant-ADP.

Fig. 2 presents the same experiment but with mant-ADP release from head 2 initiated by MgADP. The maximum rate constant observed was 1.3 s\(^{-1}\) (K\(_{1/2}\) = 0.5 μM ADP). Note that the ADP-promoted kinetics of mant-ADP release were comparable to those observed for ATP (Fig. 1), both for the observed rate of mant-ADP dissociation as well as the K\(_{1/2}\). These data suggest that the conformation required for mant-ADP release from head 2 can be achieved either by ATP binding and hydrolysis at head 1 or by ADP binding directly to head 1 to induce the structural transition.

**Is ATP Hydrolysis Required for Head 2 Mant-ADP Release?**—This experiment was performed using several nucleotides and nucleotide analogs to determine whether the post-ATP hydrolysis state at head 1 is required for the second head to bind the microtubule and release its mant-ADP (Fig. 3). The Mt-N-mant-ADP (1 μM MC1, 0.5 μM mant-ADP, 20 μM tubulin) was preformed with limiting mant-ADP with the assumption that mant-ADP would partition to the high affinity site of MC1 and the low affinity site (head bound to the microtubule) would be unoccupied. The experimental design is shown in the inset of Panel B. The Mt-N-mant-ADP complex was rapidly mixed in the stopped-flow instrument with varying concentrations of MgATP (1–100 μM). MgATP binding at the empty microtubule-bound site stimulates release of mant-ADP from the high affinity site (head 2). A, transients are shown for various ATP concentrations: 1, 2, 3, 5, 10, 20, 30, 50, and 100 μM ATP (from top to bottom transient). Each transient was fit to a single exponential function plus a linear term. B, the rate constant obtained from the exponential phase plotted as a function of microtubule concentration. Fit of the data to a hyperbola yields K\(_{a}\) = 1.4 ± 0.02 s\(^{-1}\) with half-maximal stimulation occurring at 0.64 ± 0.06 μM ATP. Because guest on July 24, 2018
AMP-PNP and ATPγS may in fact resemble an ADP-Pi intermediate to a limited extent. The small amplitude associated with the AMP-PNP and ATPγS promoted kinetics of mant-ADP dissociation is consistent with the interpretation that a post-ATP hydrolysis intermediate (either the ADP-Pi or ADP state) at head 1 is necessary for the second head to bind the microtubule and release its tightly bound mant-ADP.

ADP Release from Head 1—In order to determine the kinetics of mant-ADP dissociation from the first motor domain, MC1 was incubated with mant-ADP to replace ADP bound at the active sites of the dimer (2 mant-ADP per MC1 site, 4 mant-ADP per dimer). The MC1-mant-ADP complex was then rapidly mixed with microtubules in the absence of added nucleotide in the stopped-flow. In the absence of added ATP or ADP, microtubule-activated mant-ADP release from head 1 only is observed (Fig. 3). Fig. 4 shows the time dependence of the fluorescence change at eight different microtubule concentrations. The rate of the initial exponential phase increased as a function of microtubule concentration, and the fit of the data to a hyperbola yielded the maximum rate of mant-ADP release from the first head at 18 s⁻¹. This rate is significantly faster than the rate of mant-ADP release observed for the second motor domain at 1.4 s⁻¹ (Fig. 1). These data are consistent with the model shown in Scheme 1 in which rapid ADP release from head 1 is followed by ATP binding and hydrolysis at this site, causing the second motor domain to bind the microtubule and release its mant-ADP in the rate-limiting step of the cycle.

Tight Binding of ATP by One Head—The kinetics of mant-ADP release activated by either ATP or ADP revealed a $K_{1/2}$ of 0.5–0.6 μM (Figs. 1 and 2). These results showed that mant-ADP release from head 2 was activated at very low ATP concentrations which appeared surprising based on the $K_{m,ATP}$ at 23 μM determined by steady-state kinetics and the $K_{d,ATP}$ at 16 μM determined by the rapid quench experiments (15, 33). However, the head 2 mant-ADP release kinetics reflect ATP binding and hydrolysis to head 1 only while the steady-state and acid quench kinetics evaluate ATP turnover at both motor domains. Furthermore, because the sensitivity of the fluorescence signal is so high, very low concentrations of ATP could be used to evaluate mant-ADP release (Fig. 1). The mant-ADP release kinetics revealed a tight site for ATP binding that was not evident in our earlier experiments because their experimental design evaluated the composite behavior of both ATP-binding sites of the Ncd dimer, a tight site and a weak site.

We pursued acid quench experiments with MC1 to examine ATP binding and hydrolysis by head 1 at very low ATP concentrations, and these results are presented in Fig. 5. The time course for ATP hydrolysis was measured at 1 μM MC1 to examine the kinetics at significantly lower ATP concentrations than performed previously (15). Fig. 5A shows transients at four different ATP concentrations (2, 5, 10, and 100 μM ATP). There was an initial exponential burst of product formation corresponding to the formation of the N-ADP-Pi intermediate during the first turnover, followed by a slower linear phase which represents subsequent ATP turnovers. The rate constants determined for the linear phase of each transient were consistent with those determined by steady-state kinetics at 20 μM microtubules ($k_2$ = 1.1 ± 0.03 s⁻¹; $K_{d,ATP}$ = 16.9 ± 2.3 μM).

The rate of the initial exponential phase increased as a function of ATP concentration (Fig. 5B), and the data were fit to Equation 3. The maximum rate of ATP hydrolysis was 35 s⁻¹ and the $K_{d,ATP}$ was 3.4 μM. The burst amplitude at 40 μM ATP (Fig. 5C) was 0.5 μM (−50% of the enzyme site concentration), indicating that the data obtained at these lower ATP concentrations represents only one head. The maximum burst amplitude obtained from the fit of the data was 0.8 μM and thus representing ATP hydrolysis at both motor domains of the dimer. The $K_{d,ATP}$ at 17.8 μM determined from the burst amplitude data (Fig. 5C) is similar to the $K_{d,ATP}$ reported previously at 15 μM (15). These results indicate that the rate constants for ATP hydrolysis at head 1 (Scheme 1, $k_1$ and $k_2$) are similar, yet the ATP binding affinities at each site differ. The $K_{d,ATP}$ obtained from panel B represents $K_{d,ATP}$ for head 1 and suggests this site binds ATP tightly. The $K_{d,ATP}$ determined from the burst amplitude data (Fig. 5C) represents both sites of the dimer, implying that the second ATP molecule binds more weakly than the first. The burst amplitude at 0.8 μM rather than 1 μM is attributed to the loss of signal at very high ATP concentrations in rapid quench-flow experiments. We reported previously that the maximum burst amplitude was approximately equal to the site concentration used in the experiment (15).

Asymmetry within the Ncd Dimer—Early experiments suggested that the two sites of MC1 free in solution bound ADP with different affinities. We tested this hypothesis directly by rapidly mixing mant-ATP with dimeric MC1 in the stopped-flow in the absence of microtubules (Fig. 6). The kinetics of mant-ATP binding revealed a rapid exponential burst of fluorescence enhancement associated with mant-ATP binding to the active site, followed by a significantly slower linear phase at 0.005 s⁻¹. The rate of the exponential phase increased as a function of mant-ATP concentration, and the fit of the data to a hyperbola provided the maximum rate at 7 s⁻¹. The observation of the exponential burst in this experiment is indicative that there were MC1 sites unoccupied and available to bind mant-ATP immediately. If ADP were tightly bound at all MC1 active sites, the kinetics of mant-ATP binding would appear linear and reflect the slow release of ADP at <0.01 s⁻¹ as observed in the linear phase of the transient in Fig. 6 and reported previously for MC1 (15).

Although this stopped-flow experiment revealed unoccupied...
active sites, the amplitude of the fluorescence signal is relative and cannot be directly correlated with the concentration of MC1 active sites used in the experiment. Gel filtration experiments were performed to quantify the concentration of $[\alpha^{-32}\text{P}]\text{ADP}$ that partitions with MC1 protein. In this experiment, MC1 was incubated with $[\alpha^{-32}\text{P}]\text{ATP}$ for sufficient time to allow ADP to be released from the active site and radiolabeled ATP to bind and be hydrolyzed. The samples were then applied to a gel filtration column and centrifuged. The concentrations of MC1 and $[\alpha^{-32}\text{P}]\text{ADP}$ were determined for the excluded volume. The results in Table I show that the concentration of radiolabeled ADP that partitioned with MC1 was approximately half the concentration of MC1 protein (0.6:1). In contrast, the concentration of radiolabeled ADP that partitioned with dimeric kinesin K401 was 0.9:1 and for ovalbumin, 0.035:1. These results suggest that within the Ncd dimer, the active sites bind ADP with different affinities with one site binding ADP tightly and the other site binding ADP more weakly. The alternative interpretation that 50% of the MC1 protein is inactive appears unlikely. The protein concentration that results in one motor domain binds ADP tightly while the other head binds ADP more weakly, resulting in a rapid equilibrium at this weak site. These data suggest that upon dimerization, an asymmetry is established between the motor domains, and this asymmetry is intrinsic to the dimer before it interacts with the microtubule. Note that this asymmetry was not observed in conventional kinesin K401.

**ATP-promoted Dissociation Kinetics**—As we began to evaluate different models for the Mt-Ncd ATPase mechanism, the need to understand the point in the cycle in which the Ncd dimer completely detaches from the microtubule became critical. Previously, the rate constant for dissociation was reported at 13 s$^{-1}$ (15), yet it was unclear whether detachment of head 1 occurred at 13 s$^{-1}$ or whether the 13 s$^{-1}$ represented the dissociation of the dimer from the microtubule. As the dissociation kinetics were re-evaluated, it was apparent that the kinetics were biphasic with a rapid exponential phase, followed by a second, significantly slower exponential phase (Fig. 7). We tested the hypothesis that the initial fast exponential phase represented detachment of head 1, and the second, slow exponential phase of the turbidity kinetics represented dissociation of head 2. The experiments were repeated to analyze both exponential phases as a function of ATP (Fig. 7). As observed previously, the rate of the initial exponential phase increased as a function of ATP and was fast with the maximum observed rate constant at 12 s$^{-1}$. The rate of the second exponential phase also increased as a function of ATP concentration with the maximum observed rate of dissociation at 1.4 s$^{-1}$. These results are consistent with sequential detachment of the motor domains (Scheme 1). Although the dissociation kinetics in the second phase were observed at 1.4 s$^{-1}$, this rate constant does not necessarily represent the intrinsic rate constant. The mant-ADP release kinetics in Fig. 1 indicated that ADP release occurred at 1.4 s$^{-1}$ and was rate-limiting for the pathway; therefore, any step that occurs after this slow step ($k_a$) will be
limited by the 1.4 s \(^{-1}\) event and observed experimentally at no faster than 1.4 s \(^{-1}\).

**DISCUSSION**

**Asymmetry within the Ncd Dimer**—The mant-ATP binding kinetics and gel filtration experiments (Fig. 6, Table I) clearly indicate two sites exhibiting different affinities for ADP, one binding ADP weakly while the other binds ADP tightly. These results were really surprising because the two polypeptides are equivalent in amino acid sequence, length, and presumably state of post-translational modification because the protein is expressed in an *E. coli* expression system from a single gene. Furthermore, this behavior was never seen with the kinesin expressed in an *E. coli* expression system from a single gene.

The kinetic and gel filtration data presented here would appear to be in direct conflict with the Ncd dimeric crystal structure showing both active sites occupied by ADP (17). However, the crystallization conditions included 2 mM MgADP to stabilize the protein. Our results are completely consistent with the structural studies because the addition of 2 mM MgADP is expected to drive the equilibrium toward dimeric Ncd with both the weak and tight sites occupied by ADP. The mechanistic experiments presented here (Fig. 6, Table I) have revealed a structural intermediate that has not been detected previously and may not be detectable by conventional imaging and crystallography approaches because Ncd is labile and degrades in the absence of ADP.

The ATPase Pathway—Scheme 1 shows our model for the Mt-Ncd ATPase based on the equilibrium binding studies (33), the pre-steady-state kinetics (14, 15, 34), the motility (13, 17, 24), and structural results for Ncd (16–21, 39, 40). The cycle begins at the star (\(\star\)) intermediate, and the experimentally determined rate constants are designated. We propose that the motor domain that holds ADP more weakly (designated head 1) binds the microtubule first and stimulates fast release of ADP. The asymmetry in the Ncd dimer establishes the Mt directionality intrinsic to the Ncd dimer. Furthermore, this model predicts that dimeric Ncd takes a single step to the next microtubule binding site, yet 2 ATP molecules are required for this step and the force-generating structural transitions for...
minus-end directed movement.

There were several key experiments that excluded other potential models. The experiment presented in Fig. 1 was designed to begin as intermediate 3 with mant-ADP bound at the high affinity site. The slow rate of mant-ADP release at 1.4 s⁻¹ \((k_6)\) is the slowest step measured in the pathway and is therefore rate-limiting for steady-state turnover. Furthermore, the results presented in Fig. 3 established that ATP hydrolysis at head 1 \((k_h)\) to reach the ADP-Pi or ADP state was required for mant-ADP release from head 2 \((k_6)\). Thus, the results in Figs. 1–3 revealed the intermolecular cooperativity that was required for rate-limiting mant-ADP release.

The equilibrium binding experiments published previously (33) indicated that the only conditions that led to Ncd partitioning off the microtubule were ADP + Pi, indicating that the NADPPi intermediate was the nucleotide intermediate that detached from the microtubule. Furthermore, these data were sigmoidal, and the fit to the Hill equation indicated that two sites were cooperative. These results as well as the rapid quench burst amplitude data presented here (Fig. 5) and in Ref. 15 are indicative that both sites must hydrolyze ATP before the Ncd dimer is released from the microtubule \((k_h)\). Therefore, these experiments suggest an ATPase cycle in which both motor domains of the dimer must participate directly.

Our interpretation of the biphasic dissociation kinetics presented in Fig. 7 requires the assumption that the turbidity signal associated with intermediate 3 is greater than intermediate 6, and both are greater than the turbidity signal of the microtubule with Ncd detached and free in solution. Although at first glance this assumption may seem naive, there are several lines of evidence that support the interpretation that the second phase of the turbidity kinetics represents a true step on the pathway. Experimentally, we cannot determine a rate constant for head 2 detachment any faster than 1.4 s⁻¹ because this step is limited by ADP release at \(k_6 = 1.4\) s⁻¹. The fact that the second phase of the dissociation kinetics is ATP-dependent is indicative that this exponential phase represents a true step on the pathway rather than a nonspecific, slow linear phase seen at the end of stopped-flow transients. These are typically very slow and not ATP dependent. Furthermore, the rapid quench burst experiments show that both motor domains hydrolyze ATP during the exponential burst phase and prior to steady-state (Fig. 5). Last, the ATP-promoted dissociation kinetics for monomeric Ncd, MC6, reveal that the monomer does not detach from the microtubule. ATP-promoted dissociation requires a dimeric Ncd motor (34).

![Figure 6](http://www.jbc.org/)

**Fig. 6.** MantATP binding to MC1 in the absence of microtubules. A, MC1-ADP as purified (10 \(\mu M\)) was rapidly mixed in the stopped-flow instrument with 20 \(\mu M\) mant-ATP. The jagged line represents the fluorescence enhancement, and the smooth line is the fit of the data to the burst equation (Equation 2). The rate of the exponential phase was 3.6 ± 0.4 s⁻¹, and the rate of the linear phase was 0.05 ± 0.0006 s⁻¹. B, the observed exponential rate constant was plotted as a function of mant-ATP concentration. The data were fit to a hyperbola with a maximum rate constant of the first-order observed process at 7.0 ± 0.6 s⁻¹. Experiments at lower mant-ATP concentrations were performed with 5 \(\mu M\) MC1 (5–10 \(\mu M\) mant-ATP) and 1 \(\mu M\) MC1 (1–5 \(\mu M\) mant-ATP).

![Figure 7](http://www.jbc.org/)

**Fig. 7.** ATP-promoted dissociation kinetics of the Mt:MC1 complex. Panel A shows a representative stopped-flow transient in which the Mt:MC1 complex (5 \(\mu M\) MC1 and 4.9 \(\mu M\) tubulin, 20 \(\mu M\) taxol) was mixed with 700 \(\mu M\) MgATP (final concentrations after mixing). The smooth line shows the fit of the data to a double exponential function with the initial fast phase at 12.2 ± 0.3 s⁻¹ and the second slower phase at 1.2 ± 0.05 s⁻¹. The inset shows the ATP concentration dependence of the initial fast exponential phase. The fit of the data to a hyperbola provides the maximum rate constant at 12.2 ± 0.4 s⁻¹ which is comparable to our previously reported dissociation rate constant (15). Panel B presents the ATP concentration dependence of the second, slower exponential phase with the maximum rate constant at 1.4 ± 0.07 s⁻¹. Included in Panel B is a schematic of the experimental design. Experiments were performed at three different concentrations of Mt:MC1 complex to optimize the turbidity signal: 5 \(\mu M\) MC1 + 4.9 \(\mu M\) tubulin for 5–500 \(\mu M\) MgATP, 2.5 \(\mu M\) MC1 + 2.3 \(\mu M\) tubulin for 2.5–50 \(\mu M\) MgATP, and 1 \(\mu M\) MC1 + 1 \(\mu M\) tubulin for 0.5–5 \(\mu M\) MgATP.

**Table I**

Gel filtration results

| Protein   | Range (µM ADP/µM site) | Mean (µM ADP/µM site) |
|-----------|------------------------|-----------------------|
| K401      | 0.81–0.98              | 0.89 ± 0.03           |
| MC1       | 0.53–0.71              | 0.61 ± 0.03           |
| Ovalbumin | 0.033–0.037            | 0.035 ± 0.002         |

signal associated with intermediate 3 is greater than intermediate 6, and both are greater than the turbidity signal of the microtubule with Ncd detached and free in solution. Although at first glance this assumption may seem naive, there are several lines of evidence that support the interpretation that the second phase of the turbidity kinetics represents a true step on the pathway. Experimentally, we cannot determine a rate constant for head 2 detachment any faster than 1.4 s⁻¹ because this step is limited by ADP release at \(k_6 = 1.4\) s⁻¹. The fact that the second phase of the dissociation kinetics is ATP-dependent is indicative that this exponential phase represents a true step on the pathway rather than a nonspecific, slow linear phase seen at the end of stopped-flow transients. These are typically very slow and not ATP dependent. Furthermore, the rapid quench burst experiments show that both motor domains hydrolyze ATP during the exponential burst phase and prior to steady-state (Fig. 5). Last, the ATP-promoted dissociation kinetics for monomeric Ncd, MC6, reveal that the monomer does not detach from the microtubule. ATP-promoted dissociation requires a dimeric Ncd motor (34).
A very careful mechanistic study on dimeric Ncd was published by Pechatnikova and Taylor (14), and our kinetics are very similar to theirs. However, we have excluded their model based on our previously published dissociation kinetics (33). In the Pechatnikova and Taylor model, ATP binding at the vacant site of intermediate 3 (our Scheme 1) leads to detachment of Ncd as the ATP-ADP intermediate. This intermediate subsequently rebinds to the microtubule by the ADP-containing head, followed by ATP hydrolysis, and ADP release as the rate-limiting step. We propose that Ncd cannot detach as the ATP-ADP intermediate because ATP hydrolysis is required for dissociation (33). We performed a dissociation experiment as shown in Fig. 7, but dissociation was initiated by either ATP or the nonhydrolyzable ATP analog, AMP-PNP. In the presence of AMP-PNP, there was no change in the turbidity signal indicating that ATP binding is not sufficient to stimulate dissociation. These kinetics show that ATP binding and ATP hydrolysis must both occur for Ncd to detach from the microtubule (33).

The model presented in Scheme 1 is consistent with the mechanistic, motility, and structural results for dimeric Ncd. This model accounts for the minus-end direction of motion and reveals cooperative interactions that are important for force generation for a non-processive dimeric motor. This model is attractive because it provides a mechanism to account for the directional bias of intermediate 3 and minus-end directed Ncd motility. The determinants for minus-end directionality have been localized to the neck linker sequence, and these results evaluated in the context of the kinetics presented here lead to the testable hypothesis that the neck linker sequence establishes the asymmetry within the Ncd dimer. The sequential ATPase mechanism for Ncd is quite different from conventional kinesin’s ATPase mechanism. This study with dimeric Ncd illustrates the mechanistic diversity for energy transduction that is utilized by two kinesin superfamilly members.

Acknowledgments—We thank Dr. Sharyn Endow for the generous gift of the MC1 clone, Dr. Smita Patel for stimulating discussions, and Lisa M. Klumpp for critically reading the manuscript. In addition, we acknowledge the importance of the critical comments of our reviewers that provided insight and strengthened the manuscript significantly.

REFERENCES
1. Endow, S. A., Henikoff, A., and Soler-Niedziela, L. (1990) Nature 345, 81–83
2. Walker, R. A., Salmon, E. D., and Endow, S. A. (1990) Nature 347, 780–782
3. McDonald, H. B., and Goldstein, L. S. B. (1990) Cell 61, 991–1000
4. McDonald, H. B., Stewart, R. J., and Goldstein, L. S. B. (1990) Cell 63, 1159–1165
5. Hirokawa, N. (1996) Science 279, 519–526
6. Vale, R. D., and Fletterick, R. J. (1997) Annu. Rev. Cell Dev. Biol. 13, 745–777
7. Vale, R. D., and Milligan, R. A. (2000) Science 288, 88–95
8. Kull, F. J., Sahlin, E. P., Lau, R., Fletterick, R. J., and Vale, R. D. (1996) Nature 380, 555–559
9. Sahlin, E. P., Kull, F. J., Cooke, R., Vale, R. D., and Fletterick, R. J. (1996) Nature 380, 555–559
10. Sack, S., Muller, A., Marx, M., Thormahlen, M., Mandelkow, E.-M., Brady, S. T., and Mandelkow, E. (1997) Biochemistry 36, 16155–16165
11. Vale, R. D., Reese, T. S., and Sheetz, M. P. (1985) Cell 227, 780–782
12. Howard, J., Hudspeth, A. J., and Vale, R. D. (1989) Nature 341, 154–158
13. DeCastro, M. J., Ho, C. H., and Stewart, R. J. (1999) Biochemistry 38, 5076–5081
14. Pechatnikova, E., and Taylor, E. W. (1999) Biophys. J. 77, 1003–1016
15. Foster, K. A., and Gilbert, S. P. (2000) Biochemistry 39, 1784–1791
16. Kozielski, F., Sack, S., Marx, M., Thormahlen, M., Schonbrunn, E., Biou, V., Thompson, A., Mandelkow, E. M., and Mandelkow, E. (1997) Cell 91, 985–994
17. Sablin, E. P., Case, R. B., Dai, S. C., Hart, C. L., Ruby, A., Vale, R. D., and Fletterick, R. J. (1998) Nature 395, 813–816
18. Sosa, H., Dias, D. P., Hoenger, A., Whittaker, M., Wilson-Kubalek, E., Sablin, E., Fletterick, R. J., Vale, R. D., and Milligan, R. A. (1997) Cell 90, 217–224
19. Hoenger, A., Sack, S., Thormahlen, M., Marx, A., Muller, J., Gross, H., and Mandelkow, E. (1998) J. Cell Biol. 141, 419–430
20. Hirose, K., Lowe, J., Alonso, M., Cross, R. A., and Amos, L. A. (1999) Mol. Biol. Cell 10, 2063–2074
21. Hirose, K., Cross, R. A., and Amos, L. A. (1998) J. Mol. Biol. 287, 389–400
22. Henningsen, U., and Schiwa, M. (1997) Nature 389, 83–85
23. Case, R. B., Pierce, D. W., Hjembo, N. H., Hart, C. L., and Vale, R. D. (1997) Cell 90, 959–966
24. Endow, S. A., and Waligora, K. W. (1998) Science 281, 1200–1202
25. Rice, S., Lin, A. W., Safer, D., Hart, C. L., Naber, N., Carragher, B. O., Cain, S. M., Pechatnikova, E., Wilson-Kubalek, E. M., Whittaker, M., Pate, E., Cooke, R., Taylor, E. W., Milligan, R. A., and Vale, R. D. (1999) Nature 402, 778–784
26. Hackney, D. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6865–6869
27. Ma, Y. Z., and Taylor, E. W. (1997) J. Biol. Chem. 272, 724–730
28. Gilbert, S. P., Moyer, M. L., and Johnson, K. A. (1998) Biochemistry 37, 7962–7969
29. Moyer, M. L., Gilbert, S. P., and Johnson, K. A. (1998) Biochemistry 37, 800–813
30. Lockhart, A., Cross, R. A., and McKillop, D. F. A. (1995) FEBS Lett. 368, 531–535
31. Crevel, I. M. T. C., Lockhart, A., and Cross, R. A. (1996) J. Mol. Biol. 257, 65–76
32. Pechatnikova, E., and Taylor, E. W. (1997) J. Biol. Chem. 272, 30735–30740
33. Foster, K. A., Correia, J. J., and Gilbert, S. P. (1998) J. Biol. Chem. 273, 35307–35318
34. Maciej, A. T., and Gilbert, S. P. (2000) Biochemistry 39, 1346–1355
35. Woodward, S. K. A., Eccleston, J. F., and Geeves, M. A. (1991) Biochemistry 30, 422–430
36. Chandra, R., Salmon, E. D., Erickson, H. P., Lockhart, A., and Endow, S. A. (1995) J. Biol. Chem. 268, 9005–9013
37. Shimizu, T., Sablin, E., Vale, R. D., Fletterick, R., Pechatnikova, E., and Taylor, E. W. (1995) Biochemistry 34, 13259–13266
38. Gilbert, S. P., and Johnson, K. A. (1993) Biochemistry 32, 4677–4684
39. Arna, I., Metuz, F., DeBonis, S., and Wade, R. H. (1996) Curr. Biol. 6, 1265–1270
40. Naber, N., Cooke, R., and Pate, E. (1997) Biochemistry 36, 9681–9689
A Mechanistic Model for Ncd Directionality
Kelly A. Foster, Andrew T. Mackey and Susan P. Gilbert

J. Biol. Chem. 2001, 276:19259-19266.
doi: 10.1074/jbc.M008347200 originally published online March 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M008347200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 10 of which can be accessed free at
http://www.jbc.org/content/276/22/19259.full.html#ref-list-1