The non-claret disjunctional protein (Ncd) is a kinesin-related microtubule motor that moves toward the negative end of microtubules. The kinetic mechanism of the monomer motor domain, residues 335–700, satisfied a simple scheme for the binding of 2’-3’-O-(N-methylanthranilloyl) (MANT) ATP, the hydrolysis step, and the binding and release of MANT ADP,

\[ k_1 > 300 \text{ s}^{-1} \]
\[ k_2 = 15 \text{ s}^{-1} \]

where \( k_1 \) and \( k_2 \) are the rates of a first order step, an isomerization induced by nucleotide binding. The apparent second order rate constants for the binding steps are 1.5 \( \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \) for MANT ATP and 3.5 \( \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \) for MANT ADP (conditions, 50 mM NaCl, pH 6.9, 21°C). The rate constant of the hydrolysis step \( (k_3) \) was obtained from quench flow measurements of the phosphate burst phase corrected for the contribution of the rate of product release to the transient rate constant. The rate of phosphate dissociation was not measured; the value was assigned to account for a steady state rate of 3 s\(^{-1} \). The M\( \text{N} \) complex is dissociated by ATP at a rate of 10 s\(^{-1} \) based on light scattering measurements. Dissociation constants of M\( \text{N} \)-nucleotide complexes from microtubules increased in the order adenosine 5’-O-(thiotriphosphate) (ATP\( \gamma \)S) < ADP-ALF\( _4 \) < ATP < ADP < ADP-vanadate. Comparison of the properties of Ncd with a monomeric kinesin K332 (Ma and Taylor (1997) J. Biol. Chem. 272, 717–723) showed a close similarity, except that the rate constants for the hydrolysis and ADP release steps and the steady state rate are approximately 15–20 times smaller for Ncd. There are two differences that may affect the reaction pathway. The rate of dissociation of M\( \text{N} \) by ATP is comparable to the rate of the hydrolysis step, and N-T may dissociate in the cycle, whereas for kinesin, dissociation occurs after hydrolysis. The rate of dissociation of M\( \text{N} \) by ADP is larger than the rate of ADP release from M\( \text{N} \)-D, whereas for the microtubule-kinesin complex, the rate of dissociation by ADP is smaller than the rate of ADP release. The monomeric M\( \text{T} \)-Ncd complex is not processive.

The non-claret disjunctional (Ncd) protein is a member of the sub family of kinesin proteins, which have the motor domain at the C-terminal end of the polypeptide chain and move toward the negative end of the microtubule (1). The velocity of Ncd movement of 100–200 nm s\(^{-1} \) (2, 3) is about five times slower than neuronal kinesin. The motor domain of approximately 350 amino acid residues shows 40% homology with kinesin, and the three-dimensional structures of the Ncd and kinesin motor domains are similar (4, 5).

Previous kinetics studies (6–8) have shown that there are similarities in the reaction mechanism despite the difference in rate and polarity of movement. The dissociation of ADP is the rate-limiting step in ATP hydrolysis in the absence of microtubules, and the rate is markedly increased by microtubule binding. The rate of ADP dissociation makes a major contribution to rate-limiting the fully activated ATPase based on measurements of the maximum rate of dissociation of ADP in the reaction of the motor-ADP complex with microtubules. The ratio of the rate of ADP dissociation to \( V_m \) is approximately 1.5 for Ncd, and a similar ratio was obtained for various kinesin constructs (9, 10). However, Gilbert et al. (11) obtained a much higher rate of dissociation of ADP for a Drosophila kinesin and concluded that ADP dissociation was not rate-limiting.

The present work was undertaken to provide a more detailed kinetic scheme for an Ncd monomer and to compare the scheme with a kinesin monomer (human kinesin construct K332). A difficult problem in studying Ncd is that removal of the strongly bound ADP leads to aggregation and denaturation of the protein. The microtubule-Ncd complex is sufficiently stable in the absence of bound nucleotide to permit kinetic measurements to be made for comparison with the microtubule-kinesin complex.

The microtubule-activated Ncd is very similar to kinesin in showing a phosphate burst phase, and the hydrolysis of ATP is rate-limited by ADP release. It could be approximately described as a slow kinesin. However, the relative rates of some steps in the mechanism, including the rates of dissociation of the Mt-Ncd complex compared with the rates of the hydrolysis and ADP release steps, are significantly different from kinesin, which raises the possibility that the main reaction pathways are different for Ncd and kinesin.

MATERIALS AND METHODS

Expression and Purification of Proteins—The Ncd motor domain, Arg-335–Lys-700, was prepared from BL21 (DE3) host cells transformed by the recombinant plasmid pH40P-Ncd. The purification was described in Shimizu et al. (7). The protein was stored at –80 °C in 1. The abbreviations used are: Ncd, non-claret disjunction, construct of Arg-335–Lys-700 amino acid residues; K332, kinesin construct of 332 amino acid residues; AMP-PNP, 5’-adenylyl-β,γ-imidoediphosphate; MANT, 2’-3’-O-(N-methylanthraniloyl)-3’-deoxy-2’-MANT ATP, 2’-3’-O-(N-methylanthraniloyl)-3’-AMP; ATP-S, adenosine 5’-O-(3-thiotriphosphate); PIPES, 1,4-piperazinediethanesulfonic acid; Mt, microtubule; M\( \text{N} \), complex of an Ncd motor domain with a microtubule site.

This paper is available on line at http://www.jbc.org
Dissociation constants are expressed as tubulin dimer concentration. $K_m$, tubulin dimer concentration for half-maximum ATPase activity; $V_{m}$, extrapolated maximum ATPase rate per Ncd; $K_i$, ADP, equilibrium dissociation constant of Mt-Ncd in the presence of ADP; $s$, standard definition for dissociation constants in the presence of ADP-ALF$_4$, and ADP-vanadate. For ATP and ATP-S, the dissociation constant is a weighted average value in a steady state. Values were calculated from an unconstrained fit of averaged data from three experiments. Conditions: 25 mM PIPES, pH 6.9, 1 mM EGTA, 4 mM total MgCl$_2$ plus NaCl as indicated, 22 °C. ATPase was measured in 0.5 mM ATP; binding was measured in the presence of 2 mM ATP, 2 mM ADP, 0.5 mM ATP-S, 1 mM sodium vanadate plus 2 mM ADP, 2 mM AlCl$_3$, and 10 mM NaF (equals AlF$_4$) plus 2 mM ADP.

| $V_{m}$ | $K_m$ | $K_i$ | $K_i$ | $K_i$ | $K_i$ | $K_i$ |
|--------|-------|-------|-------|-------|-------|-------|
| 25     | 3.0   | 25    | 6     | 7     | 4     | 2.9   |
| 50     | 2.8   | 11    | 14    | 14    |       |       |

standard buffer (25 mM PIPES, pH 6.9, 1 mM EGTA, 2 mM MgCl$_2$) plus 50 mM NaCl, 10% sucrose, and 1 mol of ATP/mol of Ncd. The purification of tubulin from porcine brain and the preparation of microtubules were described in Ma and Taylor (9). Taxol at a concentration of 15 μM was present in all solutions of microtubules. [3H]Ncd was obtained by reaction with N-succinimidyl[2,3-3H]propionate (Amersham Life Science, Inc.) as described in Ma and Taylor (9).

A nucleotide-free microtubule-Ncd complex was obtained by treatment with apyrase (Grade VII; Sigma) in standard buffer plus 50 mM NaCl. An apyrase concentration of 0.003 mg/ml was sufficient to release all bound ADP in 40 min at room temperature from 40 μM Ncd-microtubule complex as assayed by the decrease in fluorescence of bound MANT ADP. Hydrolysis of ATP or ADP by this concentration of apyrase could be neglected in transient phase experiments.

Binding of Ncd to Microtubules—The binding of [3H]Ncd to microtubules was measured by a sedimentation assay. Dissociation constants were obtained by fitting data to hyperbola (error bars are standard deviations for three experiments). ○, ADP dissociation constant $K_i$; ●, ADP-vanadate, 17 μM; ▲, ADP-AlF$_4$, 4.4 μM; ▲, ATP-S, 3 μM. Conditions: 25 mM PIPES, pH 6.9, 25 mM NaCl, 1 mM EGTA, 4 mM MgCl$_2$, 2 mM ADP, or 0.5 mM ATP-S, 0.5 μM NaF, 22 °C. The sodium vanadate concentration was 1 mM. For AlF$_4$, the concentrations were 2 mM AlCl$_3$, 10 mM NaF, 15 mM NaCl.

Some properties of the Ncd—Some properties of the Ncd motor domain ATPase were reported in a previous study (7). In the absence of microtubules, the ATPase activity is 0.003 s$^{-1}$ at 25 °C, and the dissociation of ADP is the rate-limiting step. The activity is increased about 1,000-fold by microtubules. The maximum rate ($V_{m}$) obtained by fitting the dependence on microtubule concentration to a hyperbola is equal to 3.0 s$^{-1}$ in 25 mM NaCl in standard buffer. The maximum rate decreased slightly with increasing ionic strength but $K_m$ (Mt), the tubulin dimer concentration at half-maximum rate, was strongly dependent on ionic strength and increased 4-fold for an increase in NaCl concentration from 25 to 50 mM. The steady state properties are summarized in Table I.

The binding of Ncd to microtubules in the presence of various nucleotides was measured for a range of ionic strengths. Binding curves in the presence of ATP-S, ADP, ADP-AlF$_4$, and ADP-vanadate are shown in Fig. 1. Dissociation constants were obtained by an unconstrained fit of each data set to a hyperbola (Table I). For the relatively strongly bound complexes with ATP-S and ADP-AlF$_4$, the observed values of the binding reached 0.7, and the maximum values calculated from the fit are in the range 0.75–0.8, normalized to the binding in the presence of AMP-PNP. For weakly bound complexes with ADP or ADP-vanadate, the observed values are less than 0.5, and the extrapolated maximum binding was only 0.6–0.65. The dissociation constants of the complexes with ADP or ADP-vanadate would be 30% larger if the fit was constrained to a maximum binding of 0.75.

However, the order of increase in the dissociation constants is not changed by the fitting procedure. The dissociation constants increase in the order $K_{MT}<K_{ATP-S}<K_{ADP-AlF_4}<K_{ATP}<K_{ADP}<K_{ADP-vanadate}$. All dissociation constants increased markedly with increasing ionic strength. The values in 50 mM NaCl are approximately two to three times larger than in 25 mM NaCl, but the order was unchanged (data not shown).

Ncd in a strongly bound complex with microtubules appears to be more stable than free Ncd. It was noted in various binding or kinetic experiments that there was a slow loss of activity with time of storage of the protein. However, even with freshly prepared Ncd, the normalized binding curve for a Ncd-ADP complex did not extrapolate to unity. It is possible that a fraction of the protein may be converted to a state that is very weakly bound to microtubules.

Kinetic Studies of the ATPase Mechanism of Mt-Ncd—Freshly prepared Ncd contained 0.9–0.95 mol of nucleotide binding sites/mol of protein, determined by dialysis against [3H]ADP. There was a slow loss of binding sites with storage. Bound MANT ATP or MANT ADP gave a fluorescence emission of 1.6–1.7 times the value for free nucleotide. To use this enhancement for transient kinetic studies, it is necessary to remove the strongly bound ADP. All attempts to prepare active nucleotide-free Ncd were unsuccessful.
of fluorescence increase for MANT ADP. Conditions: 25 mM PIPES, pH 6.9, 2 mM MgCl₂, 50 mM NaCl, 1 mM EGTA, 22 °C.

The bound ADP can be removed by treatment with EDTA or by hydrolysis of free ADP by apyrase. The ADP was first exchanged with MANT ADP, and the time course of release was followed by measurements of the decrease in fluorescence enhancement. Addition of excess MANT ADP immediately after completion of release did allow some rebinding, but the rate of the reaction was slow, and the process appeared to be rate-limited by an isomerization of the protein. The nucleotide-free Ncd began to precipitate in 10 min, and it would no longer rebind nucleotide. Similar difficulties have recently been reported (8). Consequently, transient kinetic measurements on Ncd were limited to measuring the rate of MANT ADP dissociation by microtubules (7, 8).

The formation of a complex of Ncd with microtubules reduces the affinity for ADP, and the binding to microtubules prevented aggregation of the Ncd. Free nucleotide was first removed from the stock solution of the Ncd-ADP complex by a centrifuge column step. The addition of microtubules caused the dissociation of about half of the bound ADP if the total concentration of Ncd was less than 5 μM. Sedimentation of MtNcd and resuspension yielded a complex that could be used for transient experiments. Complete removal of bound ADP was obtained by treatment of MtNcd with a low concentration of apyrase.

Although Ncd is expected to be strongly bound to microtubules in the absence of nucleotide, only 55–65% nucleotide-free Ncd was bound to microtubules, as determined by sedimentation of a microtubule-[³H]Ncd complex. The Ncd that is not bound to microtubules is no longer active, and it does not contribute to the transient kinetics.

**Table II**

| Substrate | $k_a$ (M⁻¹ s⁻¹) | Maximum rate (s⁻¹) | Rate of second fluorescence change |
|-----------|-----------------|--------------------|----------------------------------|
| MANT ATP  | $1.5 \times 10^6$ | >300               | 25 ± 4 (d)                       |
| 3'-MANT dATP | $3.6 \times 10^6$ | >300               | 23 (d)                           |
| MANT ADP  | $2.5 \times 10^6$ | 220 ± 10           | 40 ± 4 (i)                       |

The intercept of the plot of the rate of substrate binding to the MtNcd complex gave a two-step fluorescence change, an increase followed by a decrease (Fig. 2A). The same type of biphasic signal was observed with kinesin constructs (9, 10). The apparent rate constants were obtained by fitting the data to two exponential terms. The rate constant for the increase in fluorescence varied almost linearly over the concentration range (maximum rate >300 s⁻¹); the slope defines an apparent second order rate constant $k_a$ equal to $1.5 \times 10^6$ M⁻¹ s⁻¹. Conditions are as in A; the Ncd concentration was varied to give a substrate/site ratio of at least five/one except for the lowest concentration point, which is three/one.

**Fig. 2.** The binding of MANT ATP to the MtNcd complex. A, time course of the enhancement of fluorescence. The jagged curve is the computer-generated fit to two exponential terms, rate constants 80 and 23 s⁻¹, respectively. Conditions: standard buffer plus 50 mM NaCl, 21 °C, 14 μM Ncd, 14 μM microtubules, 60 μM MANT ATP. Concentrations given in all transient experiments are final concentrations after mixing one to one in the apparatus. The MtNcd complex was treated with apyrase (0.005 mg/ml) for 40 min before the experiments. B, concentration dependence of apparent rate constants for the binding of MANT ATP. Data were fitted to two exponential terms as illustrated in A. The rate constant for the increase in fluorescence varied almost linearly over the concentration range (maximum rate >300 s⁻¹); the slope defines an apparent second order rate constant $k_a$ equal to $1.5 \times 10^6$ M⁻¹ s⁻¹. Conditions are as in A; the Ncd concentration was varied to give a substrate/site ratio of at least five/one except for the lowest concentration point, which is three/one.

**Fig. 3.** Phosphate burst phase and steady state rate of MtNcd. Measurements were made in the quench flow apparatus up to 10 s; steady state rate was also measured by hand mixing on a longer time scale. Phosphate formation is expressed as mol of phosphate/mol of Ncd corrected for 35% loss of active Ncd that did not bind to microtubules after removal of ADP. Correction for hydrolysis of ATP by apyrase was negligible during the transient phase. Data are fitted to one exponential plus a linear term (PSI plot program). The steady state rate is 0.6 s⁻¹; transient rate constant, 17 s⁻¹; corrected burst size, 0.7. The points are average values of two to three measurements. The conditions are standard buffer plus 25 mM NaCl, 26 °C, 16 μM Ncd, 20 μM microtubules, 100 μM [γ⁻³²P]ATP.
obtained by linear extrapolation to zero concentration was 7–10 s$^{-1}$ in three experiments. For the case of a simple mechanism, substrate binding, an essentially irreversible hydrolysis step and release of products, the intercept is equal to the effective rate constant of product release (discussed in Refs. 9 and 10). Since the effective rate of product release must be somewhat larger than the maximum steady state rate, a value of about 5 s$^{-1}$ was expected.

The higher value obtained for the intercept could arise because the phase of decrease in the fluorescence transient can affect the fit to one exponential term at low substrate concentrations (9). MANT ATP is a mixture of 2'- and 3'-MANT ATP isomers. It was shown for kinesin that 2'-deoxy, 3'-MANT ATP gives rise to a much larger decrease phase, whereas 3'-deoxy, 2'-MANT ATP gives essentially no decrease (9, 10). Similar behavior was obtained with Mt-Ncd. In the case of 3'-deoxy, 2'-MANT ATP, the fitting of rate constants is no longer affected by a decrease phase, and the intercept of the plot of rate constant versus substrate concentration was 5 s$^{-1}$ (data not shown). It is concluded that the kinetic data are consistent with an effective rate of product release of 5–7 s$^{-1}$. Rate constants are summarized in Table II.

The Hydrolysis Step—Mt-Ncd has a phosphate burst phase that is illustrated in Fig. 3. Nucleotide-free Mt-Ncd was obtained by treatment with apyrase. Control experiments in which microtubules plus apyrase were reacted with ATP showed no phosphate burst phase. The hydrolysis of ATP by apyrase can contribute up to 20% steady state turnover rate of Mt-Ncd. Approximately one-third of the nucleotide-free Ncd is not bound to microtubules, as shown by sedimentation of the Mt-Ncd complex, and this fraction of the Ncd has lost nucleotide binding activity. Sedimentation and resuspension of Mt-Ncd to remove inactive Ncd led to further loss of nucleotide binding activity. The presence of inactive protein does not affect the value of the rate constant of the transient phase obtained by fitting to an exponential term, but it reduces the size of the phosphate burst. The data, expressed as mol of phosphate/mol of Ncd, have been corrected for inactive Ncd.

A rate constant of 18 ± 1 s$^{-1}$ was obtained from four measurements. The size of the phosphate burst, corrected for inactive protein, was 0.7 for freshly prepared protein, but values of 0.5 were obtained with protein that had been stored frozen. Measurements at 75 and 100 μM ATP gave the same rate constant within 10%; consequently, the observed rate constant corresponds to the maximum rate.

At a microtubule concentration of 20 μM, the Mt-Ncd complex remains mainly associated in 25 mM NaCl, based on the dissociation constant (Table I). One experiment was done at 150 mM NaCl, which would give a high degree of dissociation in the steady state, and the burst rate was 13 s$^{-1}$.

Rate of Dissociation of Mt-Ncd—The dissociation of the Mt-Ncd complex by ATP was measured by the small decrease in light scattering (Fig. 4). It was necessary to average three or more traces to reduce the noise level. The rate constant is 10 s$^{-1}$, and the maximum rate was reached at 75–100 μM ATP. A larger rate of dissociation was observed with ADP, but the signal was too noisy to be analyzed satisfactorily. A dimeric Ncd, which gives a much larger change in light scattering, also showed a higher rate of dissociation by ADP than by ATP.$^2$

Rate of Binding and Dissociation of ADP—The binding of MANT ADP to Mt-Ncd gave a fluorescence increase that was

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$^2$ E. Pechatnikova and E. W. Taylor, manuscript in preparation.
fitted by two exponential terms over most of the concentration range (Fig. 5A). The concentration dependence of the rate constants is complex (Fig. 5B). The larger rate constant increased with concentration to a maximum of 220 s⁻¹. The dependence was approximately fitted by a hyperbola using all data from three experiments. However, the initial slope showed some deviation from a linear plot, and the intercept at zero concentration could be in error. The value of $k_0$ from the slope is $3.5 \times 10^6$ M⁻¹ s⁻¹.

The process with the smaller rate constant contributed more than half the amplitude at the lowest substrate concentration, but the relative amplitude decreased with concentration. For concentrations greater than 60 μM, a single exponential term gave a satisfactory fit to the data. At the lowest concentration, the rate constant is 3 s⁻¹, and it showed an S-shaped increase to a value of 40 s⁻¹.

A Mt-Ncd complex that had not been treated with apyrase gave a maximum rate for the main signal, which indicates that the system was not altered by apyrase treatment. The fluorescence signal included an extra term at a rate of about 2-3 s⁻¹ throughout the concentration range. Because only part of the ADP is dissociated in forming the Mt-Ncd complex, this step corresponds to the rate of dissociation of the bound ADP, which limits the rate of binding of MANT ADP.

The rate of MANT ADP dissociation was reported previously to be 3 s⁻¹ based on the maximum rate of the reaction of Ncd-MANT ADP with microtubules plus ATP (7). The rate of MANT ADP release was re-measured for the preparations used in the present experiments. The maximum rate was 4.2 s⁻¹ for the main signal, but there was a contribution from a step with a rate constant of 1.5 s⁻¹ that did not depend on microtubule concentration (data not shown). The latter step, which cannot be on the main ATPase pathway, is evidence for two Ncd-ADP states. Two rate constants for MANT ADP release have been reported for an Ncd dimer (8).

Conclusions

The purpose of this study is to determine the rate constants for the steps in the microtubule-Ncd ATPase mechanism and to compare the scheme with kinesin. The binding of substrate, the hydrolysis step, and the dissociation of ADP are interpreted by a simplified kinetic scheme,

$$
\begin{align*}
\text{MtN} + T & \rightarrow \text{MtNT} \quad k_1 \text{ is } 300 \text{ s}^{-1} \\
\text{MtNT} & \rightarrow \text{MtND} + \text{P} \quad k_2 = 15 \text{ s}^{-1} \\
\text{MtND} & \rightarrow \text{MtN} + \text{D} \quad k_3 = 4 \text{ s}^{-1}
\end{align*}
$$

where T, D, and P refer to nucleotide triphosphate, nucleotide diphosphate, and inorganic phosphate, respectively. MtN refers to the complex of the Ncd monomer unit with a microtubule binding site. The initial step in binding of ATP or ADP probably consists of the formation of a weakly bound collision complex, but this step is omitted for simplicity; $k_1$ and $k_{-4}$ refer to the maximum rate of the isomerization step induced by binding of the nucleotide. In the case of ADP, the binding reached a maximum rate of approximately 220 s⁻¹, and the apparent dissociation constant of the initial complex is 30 μM. For ATP, the initial binding is weaker, and a maximum rate was not obtained, $k_4 > 300 \text{ s}^{-1}$.

The observed rate of the hydrolysis transient was $18 \pm 1 \text{ s}^{-1}$. It is assumed that the hydrolysis and product release steps are essentially irreversible for the conditions of the experiments. The effective rate constant of product release is $k_{2} = k_{2}k_{4}/(k_{3} + k_{4})$. At high ATP concentrations, the phosphate burst transient reaches the maximum rate $k_{2} + k_{c}$. To determine $k_{2}$, an estimate of $k_{c}$ was obtained from the steady state rate. Since $V_m = 3 \text{ s}^{-1}$ and $V_m = k_2k_4/(k_3 + k_4)$, the maximum value of $k_2$ has to be at least 4–5 s⁻¹. Also, for the kinetic scheme, the intercept of the plot of rate constant vs. ADP concentration is equal to $k_2$ (9). The value of the intercept is about 5–7 s⁻¹, which is slightly larger than expected from the simple model.

The value of $k_2$ at the microtubule concentration used for the phosphate burst measurements is less than the maximum value. Thus $k_2$ is approximately 15 s⁻¹. In terms of the simple kinetic scheme (9), the amplitude of the burst is $(k_2/(k_2 + k_3))^3$, which gives 0.7–0.8. Observed values, after correction for the Ncd, which was not bound to microtubules, were in the range 0.5–0.7.

The rate of ADP dissociation ($k_2$) is 4 s⁻¹, determined from the maximum rate of the reaction of N-ADP with microtubules. The rate constant of phosphate dissociation has not yet been measured, but a value of $10 \text{ s}^{-1}$ for $k_3$ is necessary to account for a steady state rate of 3 s⁻¹.

The decrease in fluorescence in the ATP binding reaction appears to measure the rate of a first order step in the mechanism. The rate constant of 25 s⁻¹ is larger than the value of 18 s⁻¹ for the transient phase of phosphate formation. If the decrease in fluorescence occurred at the hydrolysis or phosphate release steps, the two rate constants should be equal. The difference might arise from errors in fitting or because MANT ATP has a slightly larger rate constant for the hydrolysis step than ATP.

The binding of MANT ADP appeared to involve two first order steps, each corresponding to an increase in fluorescence, with rate constants of 220 and 40 s⁻¹. Whereas the process with the larger rate constant has the properties expected for nucleotide binding, the significance of the slower step is not clear. The dependence of the rate constant on concentration is not expected for a sequential mechanism. In the case of monomeric kinesin, evidence was obtained for two sequential steps in ADP binding (10), but for Ncd the evidence is not conclusive because the biphasic kinetics could be explained by heterogeneity of nucleotide free Ncd.

The relative affinities of various Ncd-nucleotide states for microtubules provide information on the mechanism. The dissociation constant of N-ADP from microtubules is slightly larger than the dissociation constant in presence of ATP (Table 1). Previously a smaller value of the dissociation constant, $K_0$ADP was reported (7). It was shown in subsequent studies with kinesin (9) that the apparent dissociation constant depends on Mg²⁺ ion concentration, probably because ADP competes with MgADP for the binding site and does not dissociate the protein complex. The larger value reported here was obtained at 4 mM Mg²⁺ ion concentration (2 mM MgADP plus 2 mM MgCl₂).

The binding in the presence of ATPM1S may provide an estimate of the binding of an ATP state. However, the complex with ATP-M1S is also strongly bound. The range of values is relatively small, a factor of two to three. The trend in binding constants is in agreement with data reported by Crevel et al. (8), who proposed that the cycle alternates between a strongly bound ATP state and a weakly bound ADP state.

The binding of the N-ADP-vanadate complex to microtubules is weaker than the ADP complex, and a similar result was obtained for a monomeric kinesin (10). Because vanadate can form polymeric vanadate ions at neutral pH that might bind nonspecifically (13), the binding experiment was repeated at pH 7.8, and similar results were obtained. Preliminary binding experiments at low vanadate concentrations (to avoid the formation of polyions) showed that vanadate is not strongly bound.
to N-ADP (dissociation constant ≳ 0.1 mM) in contrast to the stable complex formed with myosin-ADP (13). The results suggest that the N-ADP-vanadate complex is an analogue of N-ADP-P$_\gamma$ which may be weakly bound to microtubules, whereas the N-ADP-AlF$_4$ complex may be an analogue of N-ATP.

Comparison of the Ncd and Kinesin Mechanisms—The kinetic schemes for the Ncd monomer and a kinesin monomer K332 (9) have the same form. To a first approximation, Mt-Ncd behaves like a slow kinesin. The rate constants of the hydrolysis and ADP dissociation steps are 15–20 times smaller for Ncd versus K332, and these steps make a major contribution to the determination of $V_m$, which is also about 20 times smaller for Ncd.

The second order rate constants for MANT ADP and MANT ATP binding and the maximum rates of the transitions ($k_+$ and $k_-$) differ by at most a factor of two for the Ncd and kinesin complexes. The value of the apparent second order rate constant of ADP binding of $2.5 \times 10^6$ M$^{-1}$ s$^{-1}$ and the rate constant of dissociation of $4 \times 10^{-4}$ s$^{-1}$ give a binding constant of $6 \times 10^6$ M$^{-1}$. This value is 10 times larger than for Mt K332. The binding constant of MANT ADP to Ncd is probably 10 times larger than for K332, based on the ratio of rate constants of dissociation. Therefore, the higher affinity for ADP appears to be the main reason for the smaller turnover rates of Ncd and Mt-Ncd compared with conventional kinesins.

At the same ionic strength, the affinities of Ncd states for microtubules are slightly larger than for K332 states. The order of increasing values of the dissociation constants is the same, although the range of values is smaller. For Mt-Ncd, the fraction of the cycle time spent in an ADP state may be larger than for Mt K332, which reduces the difference in dissociation constants in the presence of ATP versus ADP.

Any difference in the kinetic mechanisms of the two motors would have to arise from differences in ratios of rate constants that affect the reaction pathway. There are significant differences in relative magnitudes of the rate constants at two stages in the mechanism. The rate constant of dissociation of Mt K332 by ATP is $40 \text{ s}^{-1}$, whereas the rate constant of the hydrolysis step is as large as $200 \text{ s}^{-1}$ (10). Analysis of the mechanism supports the conclusion that the main reaction pathway is hydrolysis followed by dissociation of a later intermediate of the cycle.

In the case of Ncd, the rate constant of dissociation is $10 \text{ s}^{-1}$, which is similar to the rate of the hydrolysis step. Although the Mt-N-ADP state may be more strongly bound than the Mt-N-ADP state, a dissociation constant of $2–3 \mu M$ is compatible with a dissociation rate constant of $10 \text{ s}^{-1}$. Also, if dissociation follows hydrolysis, a lag should be observed in the time course of dissociation determined by the rate of the hydrolysis step. Simulation of the scheme using KINSIM indicated that a fit to the rate of dissociation with almost no lag phase required dissociation of the N-ATP state at a rate of about $10 \text{ s}^{-1}$. The question of the pathway is not settled by this evidence, but dissociation of N-ATP can occur in the cycle.

The second difference is at the ADP release step. The effective rate of dissociation of ADP from the MtK332-ADP complex is two to three times larger than the rate of dissociation of K332-ADP. Although the rate of dissociation of MtN by ADP was not measured accurately, it is larger than $10 \text{ s}^{-1}$, and therefore it is at least three times larger than the rate of ADP release. Consequently, the MtN-ADP state formed by dissociation of phosphate will usually dissociate before releasing ADP, whereas the MtK332-ADP complex will release ADP and form a strongly bound state.

The values of the rate constants of the scheme determine whether the motor is processive. Mechanical processivity is defined here as the average number of movement steps before dissociation of the microtubule-motor complex. A measure of kinetic processivity is the ratio of the maximum ATPase rate to the rate constant of dissociation of the motor by ATP, since this ratio is the average number of ATPase cycles before dissociation. This number is equal to or greater than the number of movement steps, because the coupling efficiency of ATPase cycles to movement steps may be less than one. The kinetic processivity number is also applicable to a monomeric motor, which is expected to have zero coupling efficiency.

In the case of monomeric Ncd, the ratio is 0.3; consequently, the cycle is not processive. The monomeric kinesins K332 and Drosophila DKK357 have ratios of two/four (10, 14), which indicate small kinetic processivity for these monomers. Also, the Mt-N-ADP and MtN-ATP states tend to dissociate before completing the cycle. Similar kinetic evidence for a dimeric Ncd (to be presented elsewhere) also argues against processivity.

An important problem is how two proteins with similar structures move in opposite directions along the microtubule. Reconstructions from electron micrographs appear to show a difference in symmetry of the complexes of kinesin and Ncd dimers with microtubules, which suggests that a difference in the structure of the dimers is important in determining the direction of motion (15). However monomeric kinesin produces a motion toward the positive end of the microtubule (16), which indicates that the direction is an intrinsic property of the monomer, and the same may hold for Ncd, although movement of monomers has not yet been reported. The kinetic evidence raises the possibility that the order of the dissociation versus hydrolysis steps may be interchanged in the Ncd and kinesin mechanisms. If an appropriate structural change accompanies the hydrolysis step, the reaction pathway may affect the direction of motion of the two motors. This possibility will be tested by kinetic studies of chimeras of Ncd and kinesin.

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