Expression, Purification, ATPase Properties, and Microtubule-Binding Properties of the ncd Motor Domain†

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ABSTRACT: ncd is a kinesin-related motor protein from *Drosophila* that moves in the opposite direction along microtubules to kinesin. To learn more about the ncd mechanism, ncd motor domain (R335−K700) was expressed in *Escherichia coli* and its enzymatic characteristics were studied. The ncd motor domain was purified from the cell lysate by S-Sepharose chromatography, and trace amounts of contaminants were removed by passing through a MonoQ column. The yield was 20 mg from a 500 mL culture of *E. coli*. The purified ncd motor domain exhibited an unusual UV spectrum with a broad peak around 272−275 nm, which was at least partly due to the bound nucleotide. Upon incubation with radioactive ATP, ^{3}H at adenine but not ^{32}P at γ-phosphate was retained by the protein on gel filtration, indicating it bound ADP but not ATP. Thus, like kinesin, nucleotide binding to the ncd motor domain is tight, although there is an equilibrium between the protein and free nucleotide. We also used a fluorescent ATP analogue, mantATP, for the kinetic study of ncd motor domain. MantATP was turned over by ncd motor domain slowly in the absence of microtubules, but microtubules activated the turnover to a similar extent to that of ATP. Upon incubation with ncd motor domain, the fluorescent intensity of mantATP increased at 0.005 s^{-1}, which is likely to reflect the release of endogenous ADP and incorporation of mantATP into the protein. The fluorescence intensity of the ncd motor domain having bound mantADP, likewise, decreased upon mixing with ATP, representing the mantADP release. The rate was accelerated more than 1000-fold to 3.3 s^{-1} by the presence of saturating microtubules. The profiles of the mantADP release rate and the mantATP turnover rate versus microtubule concentration were nearly identical, except that the maximal rate of mantATP turnover was 30% lower than the maximal mantADP release rate. This result suggests that mantADP release substantially contributes to the overall cycle time. We have also measured the equilibrium dissociation constants for ncd motor domain binding to microtubules in the presence of ADP [K_d(ADP) = 4−5 μM] and ATP [K_d(ATP) = 6−7.5 μM] and the apparent half-maximal microtubule stimulation of ATPase activity (5−7 μM) under an identical condition. The enzymatic and microtubule-binding characteristics of ncd motor domain reported here are similar to those of kinesin. The notable exception, however, is that K_d(ADP) for kinesin is 2−3-fold larger than K_d(ATP), which suggests the existence of a weak binding ADP state in the case of kinesin but not in the case of ncd motor domain. Such a difference could be relevant for understanding the opposite polarity of movement of these two microtubule motors.

Eukaryotes generate cytoplasmic motility using two types of filaments, actin and microtubules. Actin-based motility is driven by motors that belong to the myosin superfamily (Bement & Mooseker, 1993). Microtubular motors, on the other hand, are classified into two motor families: dynein (Gibbons & Rowe, 1965) and kinesin (Vale et al., 1985). Structurally, these two microtubule motors are quite different, having no homology in their amino acid sequences. They also move in opposite directions along microtubules; kinesin moves toward the microtubule plus (more dynamic) end and dynein toward the minus end.

Recent genetic investigations uncovered many genes which have domains homologous to the kinesin motor domain (Vale & Goldstein, 1990; Endow & Titus, 1992). Some members of this kinesin superfamily have been expressed in bacteria and then demonstrated to be motors by in vitro motility assay. One such member is ncd, the product of *ncd* gene (nonclaret disjunctional) from *Drosophila*. *ncd* gene product encodes a 700-amino acid residue polypeptide that dimerizes through an extended α-helical coiled coil, as does kinesin (Chandra

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et al., 1993). Unlike kinesin, however, the motor domain of ncd is on its C-terminal. Surprisingly, when in vitro motility produced by bacterially expressed ncd was examined, it was found that ncd moves along microtubules toward the minus end, which is the direction opposite to kinesin (Walker et al., 1990; McDonald et al., 1990).

The above finding raises the intriguing question of how the directionality of movement by microtubule motors is determined. One possibility is that the position of the motor domain in the polypeptide (opposite for kinesin and ncd) somehow determines directionality. However, such a hypothesis was ruled out by the recent experiments by Stewart et al. (1993) who found that the positions of the ncd and kinesin motor domains could be changed relative to the rest of the polypeptide without affecting the direction of movement. Hence, the determinants of directionality must reside within the ca. 350-amino acid motor domains of kinesin and ncd. Since these two motor domains are 40% identical in amino acid sequence, subtle changes in structure must determine the polarity of movement by itself.

In order to elucidate how polarity of movement is specified, it is necessary to make a detailed comparison of the biochemical and motile properties of ncd and kinesin motor domains. Previously, we compared enzymatic and motile properties of ncd, kinesin, and cytoplasmic dynein and found that ncd exhibits a substrate specificity distinctly narrower than that of kinesin (Shimizu et al., 1991, 1995). In this study, we have begun a kinetic characterization of the ncd ATPase cycle using bacterially expressed ncd motor domain. We find, in agreement with Lockhart and Cross (1994), that purified ncd motor domain contains tightly bound ADP in the active site. We also show that the rate of ADP release substantially contributes to the rate-limiting step in the microtubule-stimulated ATPase cycle as was found previously for kinesin (Hackney, 1988). Thus, despite the opposite polarity of movement, ncd exhibits many similar enzymatic properties to those of kinesin.

MATERIALS AND METHODS

Chemicals and Others. Restriction endonucleases, DNA ligase, and Taq DNA polymerase were purchased from Boehringer Mannheim; dNTP set was from Pharmacia; DNA purification kit was from Schleicher & Schuell; tryptone, yeast extract, and bacto-agar were from Difco Laboratories; ampicillin, ATP, DTT, EGTA,[1] and IPTG were from Sigma Chemical Co. MantATP was synthesized and purified according to Hiratsuka (1983). Taxol was a generous gift from Dr. M. Suffness (National Cancer Institute, Bethesda, MD).

ncd Motor Domain Expression and Purification. The clone, pET-ncd, was a generous gift of Dr. Lawrence S. B. Goldstein (University of California, San Diego, CA). The translation vector pHB40P, a derivative of pET-3a with unique *Kpn*I, *Xba*I, and *Xho*I sites being introduced between *Bgl*II and *Bam*HI, was kindly provided by Dr. Sandy Johnson (University of California, San Francisco, CA).

A DNA fragment encoding R335−K700 amino acids of the predicted 700-residue ncd protein (Endow et al., 1990) was synthesized by the polymerase chain reaction from the pET-ncd plasmid using the forward and reverse primers 5′-CTC CAT ATG CGC AAA CAG CTG CAC AAC ACG GT-3′ and 5′-CTC TCT AGA CTC TTA TTT ATC GAA ACT GCC GCT-3′, respectively. The 5′-PCR oligonucleotide consisted of *Nde*I site including initial ATG codon and corresponded to the eight amino acids (R335−V342) of the ncd protein. The 3′-PCR oligonucleotide consisted of 18 bases corresponding to amino acids S695−K700 of the ncd followed by a stop codon and *Xba*I site. Three extra bases were added to each primer at the restriction sites to enhance binding by *Nde*I and *Xba*I, respectively. The gel-purified PCR fragment was cloned into pHB40P translation vector using *Nde*I and *Xba*I restriction sites. No alteration in the inserts was confirmed by DNA sequence analysis using a T7 sequencing kit (Pharmacia P-L Biochemicals).

The recombinant plasmid pHB40P-ncd, containing the truncated *ncd* gene R335−K700, was transformed into BL21(DE3) host cells carrying the bacteriophage T7 RNA polymerase gene under the control of the IPTG-inducible lac UV5 promoter (Studier et al., 1990). Cells were grown at 22 °C in 0.5 L of LB supplemented with ampicillin (0.1 mg/mL) to $A_{600} = 0.3-0.4$ and induced by addition of IPTG to 0.3 mM and rapid shaking for 7−9 h at 22 °C. Packed induced cells were suspended in 40 mL of PB [10 mM phosphate (pH 7.2), 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors] and frozen at −20 °C. After thawing at 4 °C, lysates were made using a French pressure cell press (American Instrument Co.) and then centrifuged at 30000*g* for 30 min. The product, called the ncd motor domain herein, was present largely in soluble form when expressed in bacteria.

The following procedures for the purification of the ncd motor domain were carried out at 4 °C. The high-speed cell lysate supernatant was loaded onto a 10 mL S-Sepharose FF (Pharmacia) column that was equilibrated in PB. The column was washed with PB, and then a 150 mL linear NaCl gradient (0.1−0.4 M) was applied and the active ncd motor domain was eluted at 0.15−0.2 M NaCl from the resin. The fractions containing the ncd motor domain detected by the ATPase activity in the presence of microtubules were pooled, dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 80 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and protease inhibitors, and then loaded onto a 1 mL FPLC MonoQ column (Pharmacia). The ncd motor domain did not bind to the resin under this condition and could be purified effectively from the remaining bacterial proteins that bound to the column. Purified ncd motor domain was dialyzed against a solution containing 10 mM Pipes-Na (pH 6.9), 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 5% sucrose, and protease inhibitors and stored frozen at −70 °C.

Preparation of Tubulin. Tubulin was prepared from bovine brains by cycles of polymerization and depolymerization and DEAE-Sephacel chromatography as described

[1] Abbreviations: AMPPNP, adenylylimidodiphosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; mantADP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-diphosphate; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; mantATPase, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphatase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

by Murphy and Borisy (1975). The molecular weight of the tubulin dimer was taken as 110 000.

Binding of ncd Motor Domain to Microtubules. Binding of ncd motor domain in the presence of ATP or ADP was determined by a sedimentation method. ncd motor domain was labeled with ^3H by reaction of lysine groups with *N*-succinimidyl[2,3-^3H]propionate (Amersham) after the method of Rosenfeld and Taylor (1984). The extent of labeling was <1 mol/mol. Before use, the ncd motor domain solution was centrifuged at maximum speed for 15 min in a Beckman airfuge. Microtubules and nucleotide were added, and the mixture was again centrifuged at maximum speed in the airfuge. Approximately 10% of the tubulin remained in the supernatant, and microtubule concentration was corrected accordingly. The radioactivity in an aliquot of the supernatant was counted, and the pellet, dissolved in SDS solution, was also counted to check for recovery of the total counts. For each series of experiments at different microtubule concentrations, one centrifugation was carried out with 1 mM AMPPNP. The fractional binding was 0.85−0.95 with AMPPNP even at the highest ionic strength [0.1 M NaCl, 25 mM Pipes-Na (pH 6.9), 1 mM EGTA, and 2 mM $MgCl_2$]. The binding runs in different experiments were normalized to the binding in AMPPNP. To compare binding and microtubule activation under the same conditions, a series of experiments was performed with 2 mM ATP or ADP plus 2 mM $MgCl_2$. The hydrolysis of ATP during the binding experiment was <20%.

Assays. ATPase and mantATPase assays were performed at 20 °C in the assay mixture consisting of 0.1 M Mops-NaOH (pH 7), 2 mM $MgCl_2$, 1 mM EGTA, and 5−100 μg/mL enzyme, unless otherwise noted. The enzyme reaction was terminated by adding final 0.3 M perchloric acid, and the phosphate liberated was determined by modified malachite green assay (Shimizu & Furusawa, 1986). The time course of the reaction was monitored to calculate the enzyme activity.

Fluorescence stopped-flow measurements were carried out with a Kintek stopped flow apparatus with excitation wavelength of 355 nm at 20 °C. For the emission, a cutoff filter of 365 nm was used. For near-UV and visible light spectroscopy, a Beckman DU-650 spectrometer was used. For fluorescence measurements, a PTI delta-scan 4000 or a Perkin-Elmer MPF44 spectrofluorometer was used.

The protein concentration was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

RESULTS

Expression and Purification of ncd Motor Domain. The ncd polypeptide is highly homologous to other kinesin-related proteins in the region between amino acids 356 and 700 (Chandra et al., 1993), indicating that this is the region comprising the functional motor domain. To study the properties of this motor domain, a polypeptide corresponding to amino acids 335−700 was expressed in bacteria and purified, which we herein refer to as ncd motor domain. The majority of the expressed ncd motor domain was recovered as a soluble form in the high-speed supernatant from the bacterial lysate. The lysate was then applied to a S-Sepharose column; ncd motor domain bound to this resin, but the majority of bacterial proteins passed through under the conditions used (Figure 1). This provided a highly efficient purification step. Subsequently, the ncd motor domain-containing fractions were pooled and applied to a MonoQ column; ncd motor domain flowed through this column, while the remaining bacterial contaminants were retained by the column. Twenty milligrams of >95% pure ncd motor domain could be prepared from 500 mL culture by this 1 day procedure. The ncd motor domain behaved as an oblate-shaped particle with a molecular mass of 40 kDa in neutron-scattering studies (Mendelson et al., results to be published elsewhere), which indicates that the expressed ncd motor domain is a monomer.

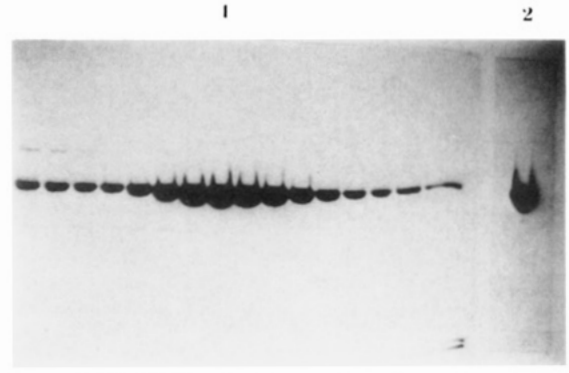

FIGURE 1: SDS−PAGE of ncd motor domain. ncd motor domain was expressed in *E. coli* and purified as described in Materials and Methods. The figure shows an SDS−PAGE pattern of the S-Sepharose elution profile (1) and that of purified protein (2); 12.5% polyacrylamide gel was used.

ATPase and Its Stimulation by Microtubules. ncd motor domain thus prepared exhibited low Mg-ATPase activity in the absence of microtubules, 0.003 s^{-1} at 25 °C, which is lower than that for the double-headed ncd (0.03−0.15 s^{-1} for several constructs; Chandra et al., 1993; Shimizu et al., 1995). This activity was stimulated by microtubules in a concentration dependent manner (cf., Figure 9). The maximal level of the activity was 1.7−2.2 s^{-1} (i.e., more than 500-fold activation) and the half-maximal activation [K_m(Mt)] was observed around 2.7 μM (0.3 mg/mL) microtubules under the conditions described in the legend to Figure 3.

The ionic and pH dependence of the ATPase activity of ncd motor domain was investigated in the presence and absence of microtubules. The pH profile in the absence of microtubules was somewhat U-shaped, reminiscent of myosin ATPase (Sekine & Kielley, 1964) or 22S dynein ATPase from *Tetrahymena* cilia (Shimizu & Kimura, 1974), but was high at neutral pH in the presence of microtubules (Figure 2A). The activity increased with increasing NaCl concentration in the assay mixture in the absence of microtubules in agreement with Lockhart and Cross (1994), but the opposite relation was observed in the presence of microtubules (Figure 2B). The apparent K_m for microtubules of microtubule-stimulated ATPase of ncd motor domain increased with ionic strength.

When the fluorescent ATP analogue mantATP, with a methylanthraniloyl group at the ribose moiety of ATP, was used as the substrate, ncd motor domain turned it over at a comparable rate to ATP (Figure 3); the basal rate was 0.001 s^{-1}, and maximal level due to microtubule stimulation was 2.3 s^{-1} at 20 °C. The half-maximal stimulation [K_m(Mt)] was observed at a similar microtubule concentration (2.7

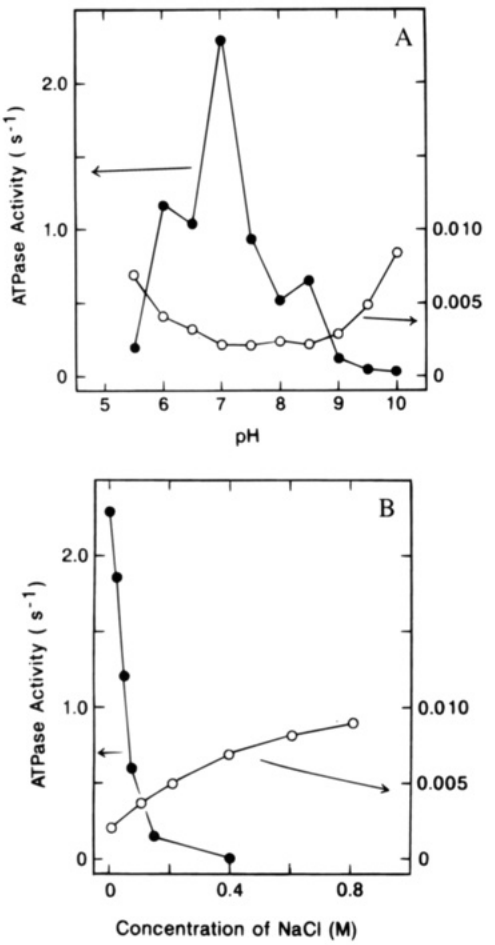

FIGURE 2: pH and NaCl concentration dependence of the ATPase activity of the ncd motor domain. (A) pH dependence. The ATPase assay was carried out at 25 °C in 90 mM buffer, 2 mM $MgCl_2$, 0.5 mM ATP, and 0.2 mg/mL ncd motor domain (○) or 0.01 mg/mL ncd motor domain plus 1.8 μM taxol-polymerized tubulin (●). For pH 5.5−6.5, Mes-NaOH, for pH 7−8, Mops-NaOH, and for pH 8.5−10, Ches-NaOH were used as buffers. (B) NaCl concentration dependence. The ATPase assay was carried out at 25 °C in 50 mM Mes-NaOH (pH 6.7), 2 mM $MgCl_2$, 0.5 mM ATP, various concentrations of NaCl as indicated in the figure, and 0.2 mg/mL ncd motor domain (○) or 0.01 mg/mL ncd motor domain plus 1.8 μM taxol-polymerized tubulin (●).

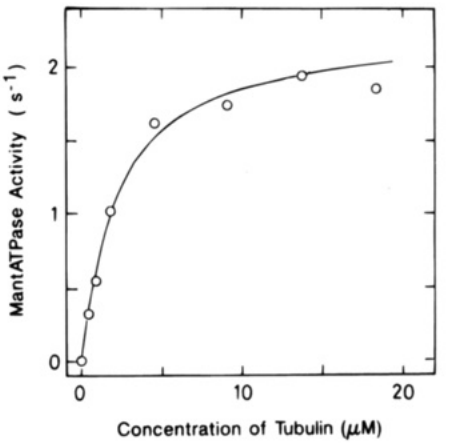

FIGURE 3: Microtubule stimulation of the mantATPase activity of ncd motor domain. The mantATPase assay was carried out in 0.1 M Mops-NaOH (pH 7), 2 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM mantATP, 0.005−0.1 mg/mL ncd motor domain, and various concentrations of taxol-polymerized tubulin as indicated in the figure. The solid line indicates a calculated hyperbola with the maximal rate of 2.3 s^{-1} and half-maximal stimulation observed at 2.7 μM taxol-polymerized tubulin.

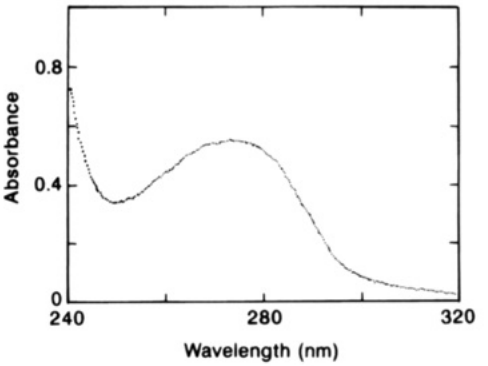

FIGURE 4: UV spectrum of purified ncd motor domain. The peak was observed at 272−275 nm. This unusual spectrum is partly due to the bound ADP: subtraction of the contribution of ADP from the whole spectrum assuming that 1 mol of ncd motor domain contains 1 mol of ADP yields a UV spectrum normal as a protein with the peak at 278−280 nm (not shown).

μM). Thus, mantATP seems to be as good an ATP analogue for the ncd motor domain as it is for kinesin (Sadhu & Taylor, 1992), myosin (Hiratsuka, 1983), and dynein (Inaba et al., 1989). Hence, we were able to utilize mantATP for a kinetic characterization of the ncd motor domain ATPase cycle.

ncd Motor Domain Contains Tightly Bound ADP. The ncd motor domain had an unusual UV spectrum for a protein, the broad peak being at 272−275 nm (Figure 4). This is partly due to a low tryptophan content [2 residues/polypeptide chain; the same number as for the kinesin motor domain (Gilbert & Johnson, 1993)]. Upon addition of perchloric acid, a substance with a UV spectrum identical with that of ATP was released in the supernatant.

To further investigate the identity of the acid-released material, the ncd motor domain was incubated with double-labeled ATP (^3H at adenine and ^{32}P at γ-phosphate) for 12 h on ice and then gel-filtrated through a prepacked 9 mL Sephadex G25 column (Figure 5). The protein peak in fraction 4 contained ^3H (3.2 μM adenine nucleotide versus 6.1 μM ncd motor domain) but little ^{32}P. This ^3H could be displaced from the protein by incubation of the peak fraction with excess cold ATP for 12 h on ice followed by the gel filtration (<0.065 mol of [^3H]adenine nucleotide remaining/mol of ncd motor domain). Our preliminary investigation on ^{31}P NMR spectra of the bound nucleotide of ncd motor domain indicates two phosphorus peaks that are different from phosphorus signals of free ADP (Y. Sukuzi, M. Tanokura, and T. Shimizu, unpublished observation). These results indicate that the ncd motor domain contains tightly bound ADP but not ATP, even after purification, which was partly responsible for the unusual UV spectrum as above (Figure 4). Although tightly bound, the ADP in the active site is in an equilibrium with free nucleotide, since it can exchange with a pool of free ATP.

MantATP Incorporation in ncd Motor Domain. Upon mixing of ncd motor domain with mantATP, the fluorescence intensity increased, indicating the binding of mantATP to the ncd motor domain. The rate was ca. 0.005 s^{-1} at 20 °C, which was close to the steady state turnover of ATP and was likely to be determined by the rate of ADP release, although the rate of mantATP incorporation might have contributed to this observed rate. After incorporation, mantATP was presumably hydrolyzed to mantADP which

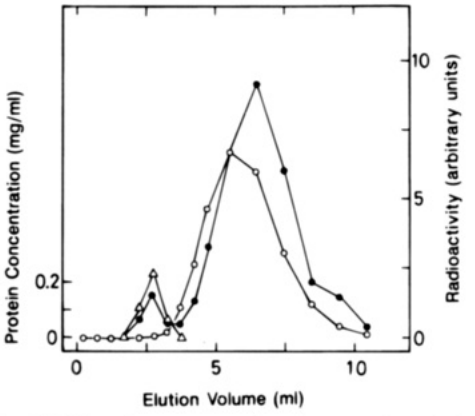

FIGURE 5: Gel filtration pattern of ncd motor domain incubated with [^{3}H,^{32}P]ATP. ncd motor domain (9 μM) was incubated on ice with 0.1 mM radiolabeled ATP for 12 h in 0.1 M Mops-NaOH (pH 7), 2 mM $MgCl_2$, 0.5 mM EGTA, and DTT and gel-filtered through a Sephadex G25 column (9 mL) equilibrated with the same buffer. After fractionation, protein concentration (△) and radioactivity (○, ●) were measured. Open circles and closed circles indicate the radioactivity of ^{32}P and ^{3}H, respectively.

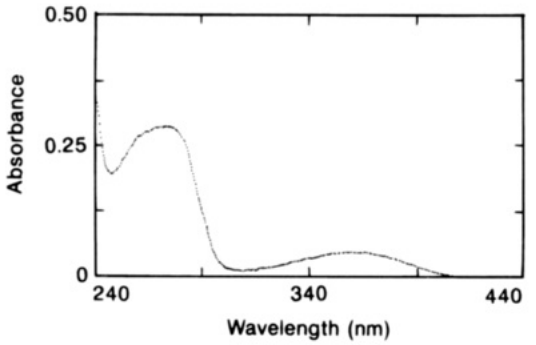

FIGURE 6: UV spectrum of ncd motor domain incubated with mantATP. ncd motor domain was incubated with excess mantATP and subsequently gel-filtered in the same way as described in the legend to Figure 5. The UV spectrum of the ncd motor domain thus prepared is shown in the figure. The broad peak at 350 nm is due to the methylanthraniloyl group of mantADP bound to the protein.

was bound tightly at the active site. ncd motor domain containing mantADP was prepared by incubating the protein with excess mantATP for 12 h on ice followed by gel filtration. The UV spectrum of the ncd motor domain thus prepared showed an absorption around 350 nm, which is indicative of a methylanthraniloyl group (Figure 6). The protein also emitted fluorescence with a peak at 450 nm, when irradiated with 350 nm light, strongly suggesting that mantADP was retained in the active site.

The fluorescence intensity of the gel-filtrated mantADP–ncd motor domain complex decreased by the addition of ATP. This was presumably due to mantADP release from and ATP incorporation into ncd motor domain. The decrease could be monitored by a fluorescence stopped-flow method (Figure 7A), and the trace was fitted with a single exponential with a rate of 0.001 s^{-1}. The rate of mantADP release was larger when microtubules were added along with ATP (Figure 7B), and the plot of the rates against the microtubule concentration could be fitted with a hyperbola (Figure 8). The maximal rate was 3.3 s^{-1}, and the half-maximal rate was observed at 3.1 μM microtubules which is similar to the concentration for half-maximal rate of mantATPase [K_m(Mt)]. The rate constant, 3.3 s^{-1}, is slightly larger than the turnover rate of 2.3 s^{-1} (values obtained with the same

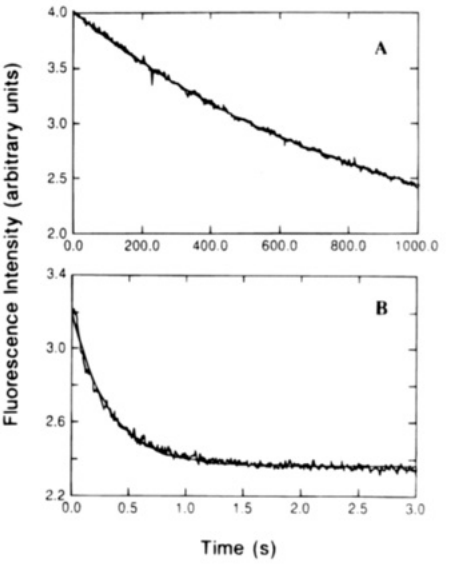

FIGURE 7: Stopped-flow analysis of decay of the fluorescence from the ncd motor domain containing mantADP. The ncd motor domain containing mantADP was prepared as described in the legend to Figure 6. It was mixed with a buffer [0.1 M Mops-NaOH (pH 7), 2 mM $MgCl_2$, and 0.5 mM EGTA] with 0.5 mM ATP and (A) 0 μM tubulin or (B) 18 μM taxol-polymerized tubulin, in a Kintek stopped-flow instrument at 20 °C. The fluorescence decay due to release of mantADP from the ncd motor domain was monitored with excitation of 355 nm and a cutoff filter of 365 nm for emitted fluorescence. The traces were fitted with single exponentials, and the best fits are shown as solid lines with rate constants of 0.0011 and 3.1 s^{-1} for panels A and B, respectively. Note that the release of mantADP in the presence of 0.5 mM ATP was accelerated by microtubules to a large extent.

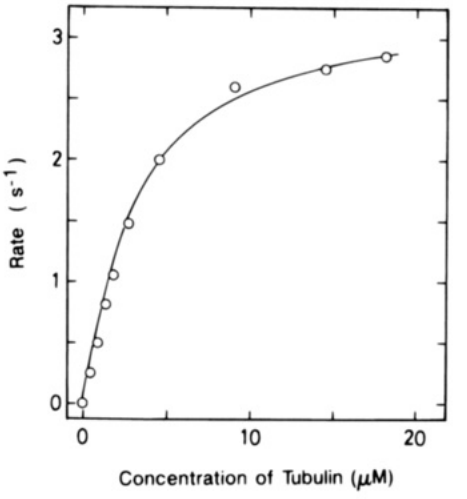

FIGURE 8: Release rate of mantADP from the ncd motor domain in the presence of various concentrations of taxol-polymerized tubulin. The rate of mantADP release from the ncd motor domain was measured as described in Figure 7, and the rate was plotted against the concentration of taxol-polymerized tubulin. The solid line is a calculated hyperbola with the maximum rate of 3.3 s^{-1} and the half-maximal effect observed at 3.1 μM tubulin.

batch of the protein within 4 h). As there are several steps in the cycle, the rate of each step must be larger than the overall cycle rate. However, this finding indicates that the mantADP release rate contributes substantially to the overall mantATP turnover rate even in the presence of saturating microtubules.

In order to ascertain the nucleotide state of the microtubule–ncd motor domain complex, ncd motor domain having mantADP was mixed with various concentrations of

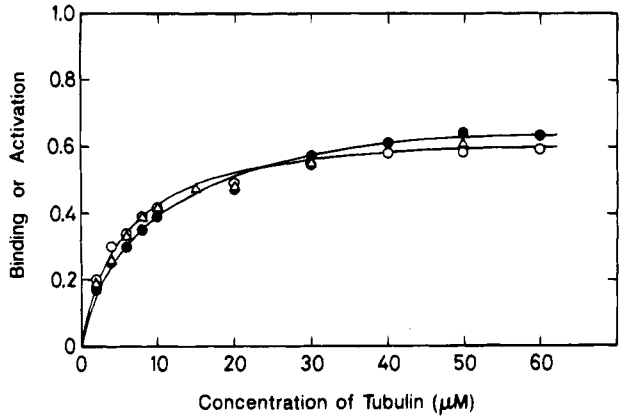

FIGURE 9: Binding of ncd motor domain to microtubules in the presence of ATP or ADP and activation of the ATPase. Binding was determined by a sedimentation assay at 20 °C as described in the text. Binding is not corrected for possible inactive protein. The solution contained 25 mM NaCl, 25 mM Pipes-NaOH (pH 6.9), 2 mM $MgCl_2$, and 1 mM EGTA. Maximum binding in the presence of AMPPNP was 0.85, and the data were normalized accordingly. Activation of ATPase versus microtubule concentration is normalized to maximum binding at the highest microtubule concentration to compare the curves (V_{max} = 2.7 s^{-1}). The dissociation constants, determined by fitting various data sets to hyperbolae, are 5, 6, and 5 μM for ADP, ATP, and microtubule activation, respectively: (○) binding in the presence of 2 mM MgADP, (●) binding in the presence of 2 mM MgATP, and (△) microtubule-stimulated ATPase.

microtubules and centrifuged, and the fluorescence intensity of the supernatant and the pellet was measured. Very little fluorescence was detected in the pellet containing the microtubule−ncd motor domain complex. This indicates that ncd motor domain loses bound nucleotide upon binding to microtubules and confirms that the signal observed with a stopped-flow apparatus was due to mantADP release.

Binding of ncd Motor Domain to Microtubules. The binding of ncd motor domain was measured in the presence of ADP and ATP for a range of microtubule concentrations. Binding curves in the presence of 25 mM NaCl are shown in Figure 9 for an ATP or ADP concentration of 2 mM. The data define the dissociation constants K_d(ADP) and K_d(ATP). The values are 5 and 6 μM, respectively, in the figure. The range of results for three preparations was 4−5 μM for K_d(ADP) and 6−7.5 μM for K_d(ATP). Thus, the dissociation constant is slightly larger for ATP, but the difference is small. The dissociation constants increased markedly with increasing ionic strength. The values in the presence of 50 mM NaCl were ca. 3 times larger, and with 0.1 M NaCl, there was a further 2−3-fold increase, although the values were too large to measure accurately.

The dependence of activation of the ATPase on the microtubule concentration under the same experimental conditions is also shown in the figure. The half-maximal stimulation [K_m(Mt)] was observed at 5 μM. The ATPase results are normalized to the same numerical value as the binding at the highest microtubule concentration to compare the curves. The actual V_{max} is 2.7 s^{-1}. The typical values for K_m(Mt) measured under such a condition (with 2 mM MgATP) were 5−7 μM.

DISCUSSION

Expression of the ncd Motor Domain. We have been able to obtain milligram quantities of highly purified ncd motor domain by bacterial expression by the 1 day procedure described herein. The amounts as well as the purity of protein that can be obtained are suitable for kinetic and X-ray crystallography studies. The microtubule-stimulated ATPase rates of ncd motor domain are similar to those described previously for larger ncd constructs containing the motor domain as well as the α-helical coiled coil rod domain (Chandra et al., 1993; Lockhart & Cross, 1994). This indicates that this truncated, monomeric form of the ncd motor has properties that are similar overall to the dimeric ncd motor, although subtle differences may exist. The ncd motor domain used in this study does not induce microtubule movement in an in vitro translocation assay. However, lack of motility may reflect an improper attachment to the glass surface, as has been suggested from results with truncated kinesin constructs (Yang et al., 1990).

Tight Binding of ADP to the ncd Active Site and Acceleration of ADP Release by Microtubules. Kinesin has been shown to contain tightly bound ADP even after purification (Hackney, 1988; Gilbert & Johnson, 1993; Huang & Hackney, 1994). In this study, we found that bacterially expressed ncd motor domain also contains ADP at the enzyme active site. Although the binding of ADP to the ncd active site is quite tight, it is in an equilibrium with free nucleotide. Hence, radiolabeled ATP or mantATP can be incorporated into the ncd active site and then hydrolyzed to the diphosphate form.

We have compared the hydrolysis rates of ATP and mantATP side-by-side and found that they are comparable. Hence, mantATP can be used as an accurate probe of kinetic steps in the ncd ATPase cycle. The rate with which mantADP is released from the ncd active site in the absence of microtubules is 0.001 s^{-1} at 20 °C, which is comparable to the steady state turnover rate of mantATP. Similar results were obtained by Lockhart and Cross (1994) using a bacterially expressed ncd construct containing the motor domain and a portion of the coiled coil stalk domain fused to glutathione *S*-transferase. In data not shown in their paper, they also stated that ADP release increased upon addition of microtubules. However, their experiments were performed at low microtubule concentrations, and it could not be determined whether another step in the ATPase pathway besides ADP release becomes rate-limiting at high microtubule concentrations. In this work, we have been able to measure mantADP release at near-saturating levels of microtubules using a stopped-flow fluorescence assay and to find that the rate of mantADP release parallels that of the mantATPase cycle. The maximum rate of mantADP release in the presence of saturating microtubules was 3.3 s^{-1}, while the V_{max} of the overall mantATPase rate was 2.3 s^{-1} under the same experimental conditions. This result indicates that ncd motor domain remains in the ADP state for a substantial amount of time during ATPase cycle in the presence of saturating microtubule concentrations. Similar to our results for ncd motor domain, ADP release from the kinesin motor domain is 1.5−2-fold faster than the V_{max} (Ma & Taylor, 1995), although Gilbert et al. (1995) reported a much larger value for this step, ca. 300 s^{-1}, with the similar kinesin motor domain.

Binding of ncd Motor Domain to Microtubules. ncd and kinesin motor domains are similar in the relations between dissociation constants in the presence of ATP and ADP and in the (large) dependence on ionic strength for the binding

Table 1: Dissociation Constants of Microtubule−Kinesin or Microtubule−ncd Motor Domain Complexes[a]

	kinesin (K379)	ncd motor domain
K_m(Mt)	2.1	6
K_d(ATP)	4.8	6.5
K_d(ADP)	13	4.5

[a] Dissociation constants were obtained under the conditions of 25 mM Pipes-Na (pH 6.9), 25 mM NaCl, 2 mM $MgCl_2$, and 1 mM EGTA at 20 °C. The nucleotide concentrations were 3 mM MgATP or MgADP for kinesin and 2 mM MgATP or MgADP for ncd motor domain. Values above are expressed in μM tubulin dimer. Definitions: K_m(Mt), tubulin concentration at half-maximal rate of microtubule-stimulated ATPase activity; K_d(ATP), that of steady state binding of a motor protein measured by centrifugation assay in the presence of ATP; K_d(ADP), same as K_d(ATP) except for the presence of ADP instead of ATP. Values for kinesin (K379) are taken from Ma and Taylor (1995), and those for ncd motor domain are averages of three measurements.

to microtubules (Ma & Taylor, 1995). An electrostatic interaction between ncd motor domain and the tubulin binding site must make a major contribution to the affinity. The magnitudes of binding constants for ncd motor domain are difficult to compare with those of kinesin because a wide range of values for binding constants and K_m(Mt) have been reported for different sources and for constructs of different length (Gilbert & Johnson, 1993; Huang & Hackney, 1994; Hackney, 1994; Ma & Taylor, 1995). It should be mentioned that K379 compared herein and the *Drosophila* kinesin constructs of 392 and 401 amino acids are dimeric, while ncd motor domain used herein is monomeric.

The values for ncd motor domain and K379 measured by the same methods are compared in *Table* 1. The dissociation constants in the presence of ADP and ATP are similar for ncd motor domain, while for K379, the dissociation constant in the presence of ADP [K_d(ADP)] is 2 or 3 times larger than that in the presence of ATP [K_d(ATP)]. The K_m for microtubule activation [K_m(Mt)] is smaller than the dissociation constant in the presence of ATP [K_d(ATP)] in the case of K379 (Ma & Taylor, 1995), and they are similar to each other for the ncd motor domain case.

Comparison of the ncd and Kinesin ATPase Cycle. By dissecting the kinetic pathways of the ncd and kinesin ATPase cycles, it is hoped that the basis of their opposite polarity of movement might become apparent. In the present work, we show that the rate-limiting step in the microtubule-stimulated ATPase cycle, at least one of them, is the ADP release, as has been previously shown with kinesin by Ma and Taylor (1995). However, a much larger rate constant of ADP dissociation was recently reported by Gilbert and others (1995). The reason for this discrepancy is not clear. In the case of kinesin (Ma & Taylor, 1995) and ncd, the rate constant of mantADP release is ca. 1.5−2-fold greater than the V_{max} for mantATP turnover. These results suggest that an enzyme•ADP state makes up at least one-half of the intermediates present in the cycle. The ncd motor domain preparation might contain some inactive protein, in which case the turnover rate per site is larger than the value reported. The rate constant for mantADP release is not affected by inactive protein which did not bind mantADP. Therefore, ADP release could make an even larger contribution to rate limiting in the cycle.

In the kinesin case, the binding to microtubules in the presence of ADP is weaker than the average binding of all the states of the cycle which implies that some states probably including Mt•K•ATP are much more strongly bound than the Mt•K•ADP state where Mt and K denote microtubules and kinesin. Romberg and Vale (1993) found that ADP dissociated the Mt•K complex in the motility assays. These results are consistent with a cycle in which the weakly bound state occurs in its latter half. This is the opposite of the actomyosin cycle in which the myosin•ADP state is more strongly bound than the ATP state, and the cycle is reset by rapid dissociation of the ADP followed by dissociation of the head by ATP [discussed in Ma and Taylor (1994)].

In the case of ncd motor domain, the binding of the ncd•ADP state is similar to or possibly slightly stronger than the binding in the presence of ATP. Since the Mt•ncd•ADP state makes a major contribution to the average dissociation constant in the presence of ATP, a larger value for K_d(ATP) could be explained by a weaker binding state which occurs earlier in the cycle. The detachment and rebinding of the motor might involve a different intermediate than in the kinesin cycle. This might be of much importance since the direction of motion could be determined by which intermediate dissociates and rebinds to reset the mechanical cycle, even if the identical "power-generating" conformational changes take place in those motor proteins as discussed by Taylor (1993) and Lockhart and Cross (1994).

Other steps in the cycle (such as nucleotide binding, hydrolysis, and phosphate release) have been difficult to dissect due to our inability to obtain a native nucleotide-free state of the ncd enzyme. We have been able to remove bound ADP from ncd motor domain by chelating magnesium ion under high salt conditions (0.5 M NaCl). Under such conditions, the ATPase activity eventually recovers when magnesium and ATP are restored, but the kinetics of ATP rebinding are very slow (0.1 s^{-1} or less with mantATP). This slow time course of nucleotide binding has also been described for a kinesin (DKH340) monomer (Huang & Hackney, 1994) and probably reflects a slow recovery of the native protein conformation. Obtaining a functional, nucleotide-free state of ncd motor domain thus represents an important challenge for the future.

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