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Comparison of ncd and Kinesin Motor Domains by Circular Dichroism Spectroscopy[1]

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ncd is a microtubule motor protein from *Drosophila*, having a 40 kDa domain homologous to the kinesin motor domain. In the present study, we investigated the circular dichroism (CD) spectra of the ncd motor domain in comparison with those of the kinesin motor domain. Although the two are about 40% identical in amino acid sequence, and recent X-ray crystallographic studies [Sablin, Kull, Cooke, Vale, and Fletterick (1996) *Nature* 380, 555-559; Kull, Sablin, Lau, Fletterick, and Vale (1996) *Nature* 380, 550-555] indicate that their core structures are nearly identical, the far UV CD spectra of ncd and kinesin motor domains, both being monomeric, were considerably different from each other, suggesting a significant difference in the secondary, especially loop structures. The motor domain of ncd, like that of kinesin, contains tightly associating ADP even after purification. We removed ADP from the ncd motor domain by gel filtration in the presence of EDTA and high salt. The resultant protein, however, was likely to be in an inactive state, since it bound ATP slowly. The far UV CD spectrum of the ncd motor domain devoid of ADP was nearly identical to that of the ncd motor domain with bound ADP. This indicated that the removal of ADP did not affect the backbone structure in the presence of high salt. On the other hand, the near UV CD spectrum of the ADP-free ncd motor domain differed from that of the ncd motor domain·ADP complex, one possibility being that the local conformation was changed upon removal of bound ADP. The near UV CD spectra of kinesin motor domain also showed a difference between the ADP-bound form and the nucleotide-free form, although the difference was much smaller.

Key words: CD, kinesin, molecular motor, ncd.

The product of the *ncd* gene (non-claret disjunctional) from *Drosophila*, ncd, is a 700 amino acid residue protein with a C-terminal globular domain that is homologous to the motor domain of kinesin, a microtubule motor protein (*1-3*). The ncd polypeptide expressed in bacteria dimerizes through its α-helical coiled-coil domain (*3*), as does kinesin, and has been shown to be a microtubule motor by *in vitro* motility assays (*2, 4*). Surprisingly, it is a microtubule minus end-directed motor, which is the opposite direction to kinesin. The amino acid residue identity between kinesin and ncd motor domains is 40%, so that subtle changes in the motor domains seem to be responsible for the reversal of the motility directionality (*5*). In addition to ncd, other kinesin-related proteins have recently been shown to be microtubule minus end-directed motors (*6, 7*).

Previously, we investigated motile and enzymatic characteristics of ncd and compared them with those of kinesin (*8, 9*). We found that ncd and an ncd derivative, GST/MC1, exhibited distinctly narrower substrate specificity in translocating microtubules than kinesin (*8*). The ncd motor domain, a monomeric C-terminal 40 kDa globular part expressed in bacteria and purified, exhibits enzymatic characteristics similar to those of kinesin (*9-14*). It contains tightly-associating ADP even after purification, the release of which is rate-limiting in the absence of microtubules. Even in the presence of saturating microtubules, the ADP release step substantially contributes to the overall ATP turnover rate (*9*). The only possible difference found so far seems to be in the microtubule-binding affinities of ncd motor domain and K379, a human kinesin motor domain dimer with a short α-helical coiled-coil segment, in the presence of ATP or ADP (*9, 14*), which might be relevant to the directionality of movement (*15-17*). On the other hand, since we did not succeed in removing the tightly associating ADP from ncd motor domain without denaturation, we were not able to measure the rates of ATP-binding and ATP-hydrolysis.

In order to learn more about the functions of kinesin and ncd, not only more detailed kinetic characterization but also structural investigations of the motor domains are important. Here we report on our study of the circular dichroism (CD) spectra of ncd motor domain. The secondary structure of ncd motor domain was estimated on the basis of these spectra. We also found that the far UV CD spectrum of the ncd motor domain was considerably different from that of the kinesin motor domain, although both are homologous.

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Abbreviations: EGTA, glycoletherdiaminetetraacetic acid; mantADP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-diphosphate; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

In addition, we removed the tightly associating ADP from the ncd motor domain with the aid of high salt, although the nucleotide-free ncd motor domain obtained was likely to be in an inactive state. We detected certain differences in the near UV CD spectra of the ncd motor domain with or without the tightly associating ADP, one possible reason for which might be a local conformational change.

MATERIALS AND METHODS

Chemicals—MantATP was synthesized and purified according to Hiratsuka (*18*). Taxol was a generous gift from Dr. M. Suffnes, National Cancer Institute, USA. ATP from Boehringer was purified by DEAE-Sephadex chromatography with a triethylammonium bicarbonate buffer (pH 7.6) gradient. Other reagents were of analytical grade.

Preparation of Proteins—The ncd motor domain was expressed in *Escherichia coli* and purified as described previously (*9*). K349, a human kinesin motor domain monomer (1-349), was prepared as described elsewhere (*19*). In brief, the high-speed supernatant of lysate of the K349-expressing *E. coli* was applied to a P-cellulose (Whatman) column, and the peak fractions of K349 were then loaded onto a Q-Sepharose (Pharmacia) column. It was further passed through an S-Sepharose (Pharmacia) resin. Tubulin was prepared from porcine brain supernatant by cycles of polymerization and depolymerization, and by DEAE-Sephacel chromatography (*20*). The molecular mass of the ncd motor domain was taken to be 41.3 kDa, and that of K349, 39.2 kDa.

Assays—ATPase and MantATPase assays were performed at 20°C in the assay mixture consisting of 20 mM Mops-NaOH (pH 7), 0.5 M NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM ATP or mantATP, and 0.005-2 mg/ml enzyme, unless otherwise noted. The enzyme reaction was terminated by adding perchloric acid to a final concentration of 0.3 M and the phosphate liberated was determined by modified malachite green assay (*21, 22*). The protein concentration was measured by the method of Lowry *et al.* (*23*), using bovine serum albumin as a standard.

Spectroscopic Measurements—Fluorescence stopped-flow measurements were carried out with a Kintek stopped-flow apparatus SF2001 with an excitation wavelength of 355 nm at 20°C. For the emission, a cut-off filter of 410 nm was used. For CD measurements, a Jasco J-600 CD spectropolarimeter was used with a temperature controlled cuvette-holder at 20°C. Prior to the measurements, the protein solutions were clarified by centrifugation at $14,000 \times g$ for 10 min; no turbidity development was detected after the measurements. For estimation of secondary structures, the CONTIN program was used (*24*). For near UV and visible light spectroscopy and for fluorescence spectroscopy, a Beckman DU-70 spectrophotometer and a Shimadzu RF-5000 were used, respectively.

RESULTS

Far UV Circular Dichroism Spectra of ncd Motor Domain and the Comparison with Kinesin Motor Domain, K349—First, we confirmed that in both the near and far UV region, ADP (complexed with Mg ion or free) gave negligible peaks and troughs in the CD spectra when compared with those of the proteins at the same concentrations as those of the proteins used in our study (up to 25 μM); one molecule of ncd motor domain or K349, a monomeric human kinesin motor domain, contains one ADP.

The far UV CD spectrum (196-260 nm) of the ncd motor domain with the tightly associating ADP in the presence of Mg ion and high salt is shown in Fig. 1. The secondary structures were predicted from the spectrum with the CONTIN program (*24*); 43% α-helix, 32% β-structure, and 25% others. The far UV CD spectrum of the ncd motor domain with bound ADP in the presence of 0.1 M NaCl was nearly identical to that in the presence of high salt, as seen in Fig. 1.

To our surprise, the far UV CD spectrum of K349 was considerably different from that of ncd motor domain, its 222 nm trough being as deep as the 208 nm trough (Fig. 2). This difference was consistently observed with four different preparations of K349 as well as of ncd motor domain. The α-helical content was estimated to be 30%, β-structure and others being 35 and 35%, respectively, in the same manner as above. The far UV CD spectrum of K349 with bound ADP in the presence of 0.1 M NaCl was very similar to that in the presence of high salt.

Preparation of Nucleotide-Free ncd Motor Domain—The ncd motor domain as purified contains tightly associating ADP (*9*), as does kinesin (*25*). We found that this ADP could be removed by passing through a prepacked gel exclusion column (10DG, BioRad) in the presence of 2 mM EDTA and 0.5 M NaCl without any indication of aggregation. In the absence of a high ionic strength, the protein was eluted from the column as aggregates, probably because of denaturation following the removal of ADP by chelating Mg ion with EDTA. Once the protein was aggregated, it was not resolubilized by adding Mg ion or Mg ion plus ATP. High salt, however, seemed to stabilize the protein and prevent denaturation even in the presence of EDTA, though it was ineffective in resolubilizing the protein aggregates once formed.

The ncd motor domain was previously shown to have an unusual UV spectrum with a broad peak at 272 to 275 nm, which was likely to be partly due to the absorption by the bound ADP (*9*). In contrast, the protein obtained as above exhibited a typical protein UV spectrum with a sharp peak at 277 nm and a deep trough at 250 nm. The nucleotide-free state of the protein was confirmed in two ways. (i) The ncd motor domain incorporated and hydrolyzed mantATP, a fluorescent ATP analogue, and the mantADP was bound tightly, as seen previously (*9*). This fluorescent nucleotide was, however, lost from the protein during gel filtration in the presence of high salt and EDTA. The resultant protein emitted little fluorescence upon excitation at 355 nm. (ii) Upon addition of perchloric acid, the ncd motor domain releases ADP, which can be detected by measuring the UV spectrum of the supernatant (*9*), whereas the ncd motor domain, prepared as above, did not release any substance with absorption in the UV range.

The far UV CD spectrum of the nucleotide-free ncd motor domain in the presence of 0.5 M NaCl was nearly identical to that of normal ncd motor domain with bound ADP, as seen in Fig. 1. This indicates that the backbone structure of the ncd motor domain did not change upon removal of the bound ADP. In addition, the far UV CD spectrum of nucleotide-free K349 prepared in the same

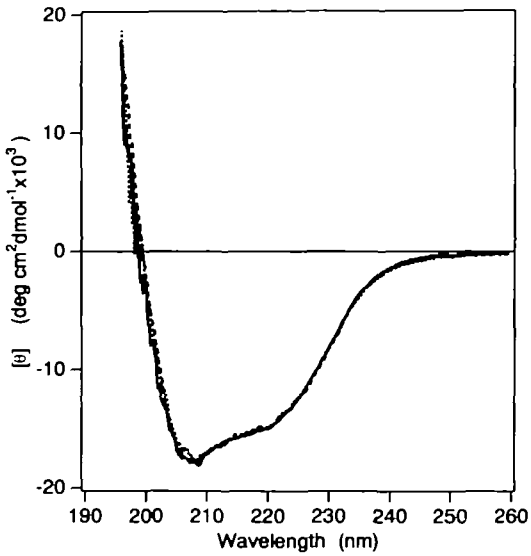

Fig. 1. **Far UV CD spectrum of the ncd motor domain.** A CD spectrum between 196 and 260 nm of ncd motor domain having bound ADP in the presence of 0.5 M NaCl and 2 mM $MgCl_2$ (solid line) was recorded with a CD spectropolarimeter (J600, Jasco) at 20°C using a 0.2 mm light-path cuvette. The secondary structure as estimated with the CONTIN program (*24*) was α-helix, 43%; β-structure, 32%; others, 25%. The figure also shows the spectrum of the nucleotide-free ncd motor domain in the presence of 0.5 M NaCl and 2 mM EDTA (dotted line), and that of ncd motor domain with bound ADP in the presence of 0.1 M NaCl and 2 mM $MgCl_2$ (broken line). The other ingredient of the solution was 20 mM Mops-NaOH (pH 7.0). The concentration of the ncd motor domain was 0.83 mg/ml (0.02 mM). A blank solution with 0.02 mM ADP exhibited negligible peaks and troughs. Mean residue ellipticities, [θ], are expressed on the basis of a mean residue weight of 113.2.

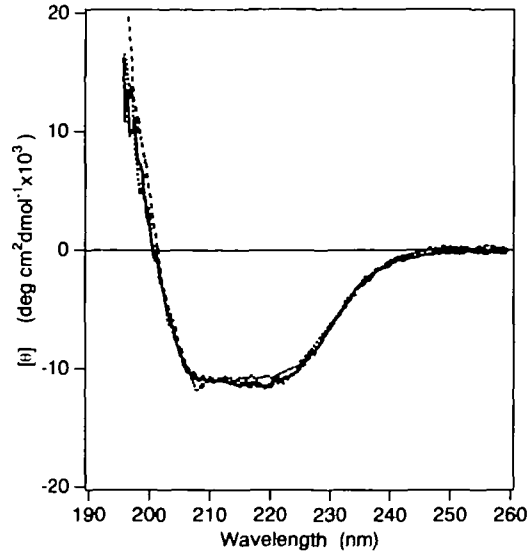

Fig. 2. **Far UV CD spectrum of K349.** A CD spectrum between 196 and 260 nm was recorded as described in the legend to Fig. 1 except that K349 in the presence of 0.5 M NaCl and 2 mM $MgCl_2$ was investigated (solid line) instead of the ncd motor domain. The secondary structure was estimated to be α-helix, 30%; β-structure, 35%; others, 35%. The figure also shows the spectrum of the nucleotide-free K349 in the presence of 0.5 M NaCl and 2 mM EDTA (dotted line), and that of K349 with bound ADP in the presence of 0.1 M NaCl and 2 mM $MgCl_2$ (broken line). The concentration of K349 was 0.78 mg/ml (0.02 mM). Mean residue ellipticities, [θ], are expressed on the basis of a mean residue weight of 112.3. Other conditions were the same as those described in the legend to Fig. 1.

manner as above was almost superimposable on that of ordinary K349 in the presence of high salt (Fig. 2).

Near UV CD Spectra of ncd Motor Domain and K349 with or without Bound Nucleotide—On the other hand, the near UV CD spectrum (240-310 nm) of the ncd motor domain in the presence of 0.5 M NaCl differed depending on the nucleotide state, as seen in Fig. 3. With the nucleotide-free ncd motor domain in the presence of EDTA, the spectrum was almost flat between 255 and 280 nm, and this was not affected by the addition of Mg ion at a higher concentration than that of EDTA. On the other hand, the ncd motor domain·ADP complex in the presence of Mg ion exhibited a broad trough in this region, the negative peak being at 261 nm. In addition, the spectrum showed a peak and a trough in the 280 to 295 nm range, which were thought to be due to tryptophan residue(s). Thus, although the backbone structure did not seem to change, the near UV CD spectra suggested that the local structure might change, depending on the nucleotide state. The CD spectrum of the ncd motor domain with bound ADP in the presence of low salt (0.1 M NaCl) was also very similar to that in the presence of high salt (Fig. 3).

When the ncd motor domain without bound nucleotide was incubated with 1 mM MgATP on ice for 20 h, the near UV CD spectrum was restored to a considerable extent, though not completely to the level of ncd motor domain·ADP; when the trough at 261 nm was compared, the restoration was 75%. This might mean that 25% of ncd motor domain was irreversibly damaged while 75% recovered the original conformation. It should be mentioned that even when the incubation period was prolonged to over 20 h, the restoration level did not change.

As shown in Fig. 4, the nucleotide-free K349 exhibited a different near UV CD spectrum from that of K349·ADP complex, as in the case of the ncd motor domain. However, the difference was smaller than that of the ncd motor domain. The far UV CD spectrum of the nucleotide-free K349 (Fig. 2) was similar to that of K349·ADP with a slightly shallower trough at 222 nm, indicating that the backbone structure was little, if at all, affected by the nucleotide state in the presence of 0.5 M NaCl, as in the case of the ncd motor domain.

Early Phase of ATP Turnover by Nucleotide-Free ncd Motor Domain—Myosin and dynein show an initial burst of phosphate production (*26, 27*); these ATPases bind and hydrolyze ATP rapidly, but release the products slowly. Hence, 1 mol of ATP per ATPase site is hydrolyzed rapidly while subsequent turnovers are slow. Kinesin was shown to exhibit the burst only after the removal of its associating ADP (*13, 28, 29*), or after complexation with microtubules (*11, 14*). Thus, we examined whether the nucleotide-free ncd motor domain prepared as above would exhibit an initial burst of phosphate production.

As shown in Fig. 5, the nucleotide-free ncd motor domain exhibited only a very small burst of phosphate production, if any, in the presence of 0.5 M NaCl. This result indicated that the nucleotide-free ncd motor domain hydrolyzed even the first ATP molecule only slowly. Figure 5 also shows the time course up to 120 s with normal ncd motor domain, *i.e.*,

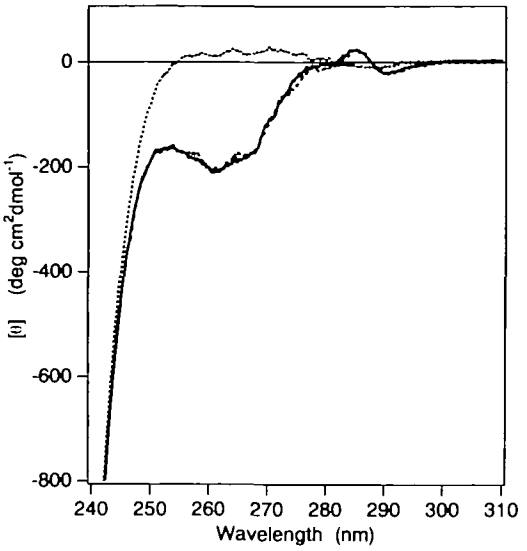

Fig. 3. **Near UV CD spectra of the ncd motor domain.** CD spectra between 240 and 310 nm were recorded as described in the legend to Fig. 1, except that a 10 mm light-path cuvette was used. The solid and dotted lines indicate the spectrum of the ncd motor domain with bound ADP in the presence of 0.5 M NaCl and 2 mM $MgCl_2$, and that of the nucleotide-free ncd motor domain in the presence of 0.5 M NaCl and 2 mM EDTA, respectively. The broken line indicates the spectrum of ncd motor domain with bound ADP in the presence of 0.1 M NaCl and 2 mM $MgCl_2$. Other conditions were the same as those described in the legend to Fig. 1.

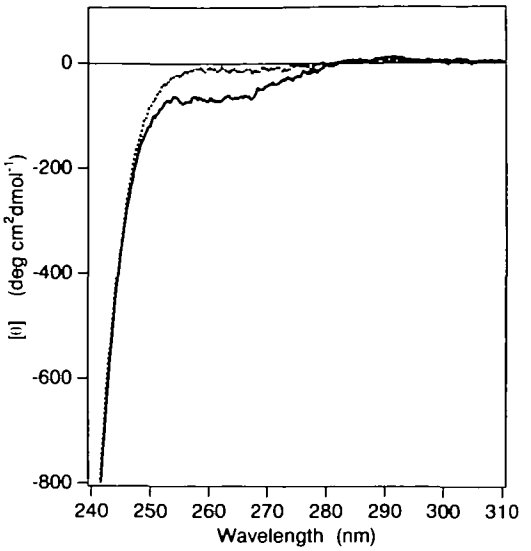

Fig. 4. **Near UV CD spectra of K349.** CD spectra between 240 and 310 nm were recorded as described in the legend to Fig. 3, except that K349 in the presence of 0.5 M NaCl was investigated instead of the ncd motor domain. The solid and dotted lines indicate K349 with bound ADP in the presence of 2 mM $MgCl_2$, and the nucleotide-free K349 in the presence of 2 mM EDTA, respectively. Other conditions were the same as those described in the legend to Fig. 2.

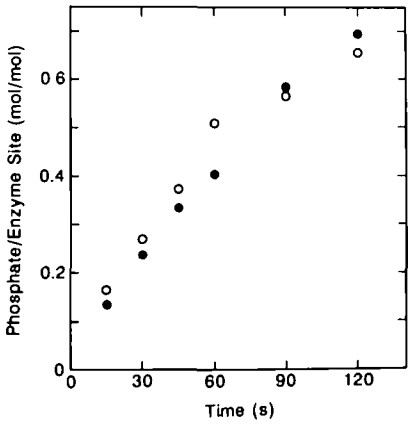

Fig. 5. **Time course of phosphate production by the ncd motor domain.** Nucleotide-free (EDTA-treated) ncd motor domain was prepared as described in "RESULTS." The assay solution contained 20 mM MOPS-NaOH (pH 7), 0.5 M NaCl, 2 mM $MgCl_2$, 0.5 mM ATP, and 2 mg/ml (0.05 mM) nucleotide-free ncd motor domain. After incubation at 20°C for 5 min, ATP was added to start the reaction. An aliquot was withdrawn at various times as indicated on the abscissa and mixed with perchloric acid (final concentration 0.3 M) to stop the reaction. The phosphate produced was determined by a modified malachite green method. For comparison, the ncd motor domain having bound ADP, *i.e.*, non-treated, was assayed in the same manner. Open symbols, nucleotide-free ncd motor domain; closed symbols, non-treated one. Note that the phosphate burst, if any, should be less than 0.05 mol/mol ncd motor domain.

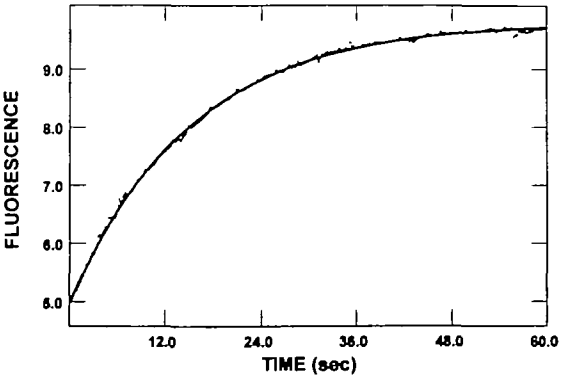

Fig. 6. **A fluorescence stopped-flow trace upon mixing the nucleotide-free ncd motor domain with mantATP.** Nucleotide-free ncd motor domain was mixed with mantATP in a stopped-flow apparatus (SF2001, Kintek), and the fluorescence intensity upon excitation at 355 nm was monitored. The solution after mixing contained 20 mM MOPS-NaOH (pH 7.0), 0.5 M NaCl, 2 mM $MgCl_2$, 0.083 mg/ml (2 μM) ncd motor domain, and 5 μM mantATP. The fluorescence increase indicates the binding of mantATP to the enzyme, and the solid line is a fitted exponential of 0.07 s^{-1}.

with bound ADP, the rate of which was 0.007 s^{-1}. This value is compatible with that in our previous report (*9*).

Next, the binding of mantATP, a fluorescent ATP analogue widely used to study ATP-metabolizing enzymes (*18*) including ncd motor domain (*9*), to the nucleotide-free ncd motor domain was investigated by the fluorescence stopped-flow method in the presence of 0.5 M NaCl. Upon mixing of the protein with mantATP, the fluorescence intensity increased, as seen in Fig. 6, but the rate was low, 0.07 s^{-1}. The rate did not increase upon increasing the

mantATP concentration from 2 to 15 μM (final concentration after mixing). This result indicated that the nucleotide-free ncd motor domain bound ATP (mantATP) slowly, but that the slow binding was still faster than the hydrolysis of ATP. Possible interpretations of these results will be discussed below. On the other hand, the fluorescence increase upon mixing of mantATP and ordinary ncd motor domain with bound ADP was 0.01 s^{-1} under identical conditions. This result is also consistent with our previous conclusion that with ncd motor domain·ADP, the ATP (or mantATP) binding is rate-limited by the ADP release step, and that the ADP (or mantADP) release step is slightly faster than the overall ATPase (or mantATPase) (*9*).

We examined the microtubule-stimulation of the ncd motor domain ATPase at 0.1 M NaCl. The ATPase reactions were started by the addition of the enzyme. The results indicated that the ATPase activity of the nucleotide-free ncd motor domain was stimulated by microtubules to a large extent, but that the stimulation level was 20 to 25% lower than that of non-treated ncd motor domain. It should be noted that there was no detectable lag even in the first 2 min of the ATPase reaction. These results suggested that about 75 to 80% of nucleotide-free ncd motor domain was potentially active and was restored to the active state rapidly in the presence of microtubules (and ATP).

DISCUSSION

Nucleotide-Free ncd Motor Domain—We previously investigated the ATPase kinetics of the ncd motor domain, and showed that ADP release is rate-limiting in the absence of microtubules, and that even in the presence of nearly saturating microtubules, the enzyme·ADP state substantially contributes to its cycle time (*9*). However, the ATP-binding rate and ATP-hydrolysis rate were undetermined, because we were not able to obtain nucleotide-free ncd motor domain. Gel filtration chromatography in the presence of low salt and EDTA, which was successful in removing ADP from bovine brain kinesin (*28, 29*), was not effective, denaturing the ncd motor domain. Binding of the ncd motor domain to S-Sepharose and eluting it in the presence of EDTA with high salt did not yield active protein, either (Shimizu, unpublished).

In the present study, we succeeded in removing ADP from the ncd motor domain without aggregation by gel exclusion chromatography in the presence of EDTA and high salt. However, the ADP-free ncd motor domain thus prepared bound ATP (mantATP) only slowly and exhibited very little, if any, phosphate burst, suggesting that it was in an inactive state (see below). In addition, the near UV CD spectra and the microtubule-stimulated ATPase results suggested that 20 to 25% of the nucleotide-free ncd motor domain had irreversibly lost its functions. Thus, we still need to explore methods to obtain nucleotide-free and active ncd motor domain for future ATPase kinetic studies. It should be noted that the full-length ncd expressed in bacteria also exhibited little phosphate burst after the removal of bound ADP by gel filtration in the presence of EDTA and high salt (Shimizu, unpublished). In addition, bacterially-expressed kinesin derivatives devoid of the tail and most of the rod domain could be converted into nucleotide-free forms by the same gel filtration method, but those nucleotide-free kinesin derivatives bound ATP slowly, perhaps being inactive (*30*; Shimizu, unpublished). The only exceptions are kinesin prepared from brains and gel-filtered in the presence of EDTA (*28, 29*), and K379 adsorbed on P-cellulose and eluted with EDTA and high salt (*13*).

The fluorescence change upon mixing of mantATP with nucleotide-free ncd motor domain was slow, 0.07 s^{-1} (Fig. 6); yet this rate was about ten times faster than the phosphate production rate (Fig. 5). This fluorescence change may represent the transition from inactive to active state of the ncd motor domain devoid of bound ADP, this active state being able to bind ATP or mantATP rapidly. It is thought to be less likely that this slow fluorescence change is due to a slow conformational transition after rapid binding of mantATP to the active site of the ncd motor domain, because the fluorescent group of mantATP is sensitive to the environment, especially hydrophobicity, so that if it is bound to protein, the fluorescence property should be immediately influenced by the protein.

The even slower phosphate production could be due to a conformational transition after binding of ATP (or mantATP) with fast hydrolysis, or due to the slow hydrolytic nature of the ncd motor domain as an ATPase. With kinesin, it has been shown that the hydrolytic step is fast in the absence of microtubules (up to 10 s^{-1}) but that this step can be accelerated by microtubules (*11, 13, 14*).

CD Spectra of ncd Motor Domain and the Comparison with Those of K349—Circular dichroism spectroscopy is extremely effective in detecting conformational changes in proteins and in estimating secondary structures. We were interested in detecting possible structural changes associated with the removal of the bound nucleotide from the ncd motor domain.

Although we detected little change in the far UV CD spectra upon ADP removal from the ncd motor domain, substantial differences in the near UV CD spectra were observed. This suggests that although the backbone structure was little changed upon ADP removal, the local structure was altered. It should be noted that although free ADP in solution showed negligible CD activity, we can not exclude the possibility that ADP itself contributed to the near UV CD spectrum by its binding to the protein. We are now in the process of investigation of such a possible contribution of ADP itself by utilizing several ADP analogues. Further, we could not compare the ncd motor domain complexed with ADP with nucleotide-free and active ncd motor domain, since we have been unable to obtain the latter species.

Recently, the three-dimensional structures of the ncd motor domain and K349, the same constructs as those used in this study, have been solved (*31, 32*). According to those reports, the core structures are remarkably similar to each other, including six α-helices and eight β-strands, while the loop structures may be different between the two. It was surprising, therefore, to find that K349 showed a far UV CD spectrum that is quite different from that of the ncd motor domain. Those studies and our far UV CD spectral data suggest that the difference in CD spectra probably reflects loop differences, since the far UV CD spectra could be considerably influenced by loop regions of proteins. The secondary structure prediction with the aid of the CONTIN program (*24*) gave larger values for α-helix and β-strand contents for both ncd and kinesin motor domains; from

X-ray structural analyses, the α-helix and β-strand contents of the ncd motor domain are 30 and 25%, and those for K349 are 28 and 25%. Such differences may not be uncommon with some proteins.

Although the core structures are very similar, the difference in the loop structures may be significant, and may be relevant to the difference in the motility directionality (*31, 32*).

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