J. Biochem. **138**, 95–104 (2005)
DOI: 10.1093/jb/mvi104

Removal of Tightly Bound ADP Induces Distinct Structural Changes of the Two Tryptophan-Containing Regions of the ncd Motor Domain

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Received April 11, 2005; accepted May 10, 2005

ncd is a molecular motor belonging to the kinesin superfamily. In solution, it is a homo-dimer of a 700 amino acid polypeptide. The C-terminus of each polypeptide forms a globular domain of about 40 kDa, the motor domain with ATPase activity. The ATPase site of the motor domain of kinesin family members, including ncd, binds ADP tightly, the release of which is facilitated by microtubules during the mechanochemical ATPase cycle. Previously, we studied the spectroscopic characteristics of the ncd motor domain, focusing on interactions of the transition-moment-dipoles between ADP and aromatic amino acid side chains using circular dichroism (CD) spectroscopy. In the present study, we generated several ncd motor domain mutants. In each, a tryptophanyl or specific tyrosyl residue was mutated. We found that Trp370 and Tyr442, the latter of which stacks directly with the adenine moiety of bound ADP, caused the bound ADP to exhibit peculiar CD signals. In addition, fluorescence measurements revealed that Trp370, but not Trp473, was responsible for the emission intensity change depending on the presence or absence of bound ADP. This fluorescence result implies that the structural change induced at the ADP-binding site (on the release of the ADP) is transmitted to the region that includes Trp370, which is relatively close to the ADP-binding site but not in direct contact with the ADP-binding region. In contrast, Trp473 in the region that is in contact with the α-helical coiled coil stalk did not experience the structural changes caused on removal of ADP. The distinct behavior of these two tryptophanyl residues suggests that the ncd motor domain has a bifacial architecture made up of a relatively deformable side including the nucleotide binding site and a more rigid one.

Key words: ADP-binding, CD spectroscopy, conformational change, fluorescence, molecular motor, ncd.

Abbreviations: DTT, dithiothreitol; EGTA, glycoletherdiaminetetraacetic acid; MOPS, 3-morpholinopropanesulfonic acid; WT, wild type.

Kinesin and its family member ATPases are molecular motors that move along microtubules with some exceptions (*1–4*). As motor enzymes, kinesin and its family members have common characteristics in terms of ATPase kinetics (*5–7*)—the basal ATPase activity is very low, but is dramatically stimulated (1,000 fold or more) by microtubules. Another important characteristic is the tight binding of ADP at the nucleotide binding site (*5*, *8*).

The three-dimensional structure of the motor domain of kinesin, whose molecular mass is ca 40,000, and those of some other family members have been solved (*9–15*). One remarkable feature of the structures is that the adenine moiety of tightly bound ADP stacks directly with an aromatic amino acid residue, which may explain the tight binding of ADP. For ncd, a molecular motor from *Drosophila melanogaster*, this residue is tyrosine (Tyr442), while in human kinesin, it is histidine (His93).

The ncd motor domain (Fig. 1) has three major aromatic amino-acid residue clusters: (Trp370, Tyr372, His403), (Phe402, Phe644, Phe352) and (His565, Trp473, Tyr475, Tyr468). The first and third clusters each contain one of the only two tryptophanyl residues of the ncd motor domain. A six-stranded β-sheet exists at the core of the motor domain, and Trp370, Trp473 and ADP are at the periphery of this core. The distance from ADP is about 1.5 nm and 3.8 nm for Trp370 and Trp473, respectively. Trp473 and Tyr475 are likely to stack with each other while Trp370 and Tyr372 are not. In addition, Trp370 is not conserved among kinesin superfamily members while Trp473 (as well as Tyr475) is well conserved among C-terminal motors.

The removal of the tightly bound ADP from kinesin was successfully performed with EDTA treatment by Hackney *et al.* (*16*). With the ncd motor domain, the

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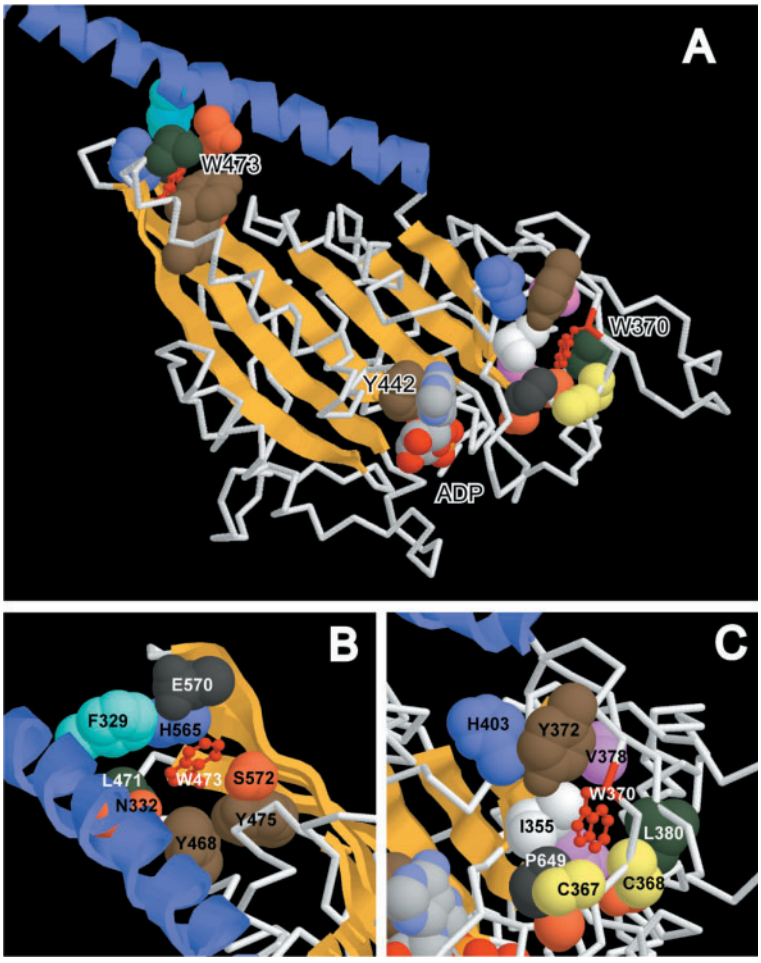

Fig. 1. **Graphic presentation of mutation sites in relationship to bound ADP.** The X-ray crystallographic data, PDB accession code 1CZ7, were processed to show the structure using Rasmol software. The ncd motor domain is graphically shown together with the extended α-helical coiled-coil stalk colored bluish purple (A). In the current study, a short construct (335–700) was used, which does not have most of the α-helical coiled-coil stalk portion. The motor domain has a six-stranded β-sheet as the core, and the amino acid residues concerned, Trp370, Trp473 and Tyr442, and the bound ADP are highlighted. The surrounding structure is also shown. The lower figures (B and C) zoom in on the two hydrophobic regions, containing Trp473 and Trp370, respectively.

removal could only be performed in the presence of high salt; with low salt, the ncd motor domain precipitates instantaneously upon the addition of EDTA and is never resolubilized. On gel filtration in the presence of 0.5 M NaCl and 2 mM EDTA, we obtained an ADP-free ncd motor domain that retained important structural features, at least for some time (*17*).

We were interested in whether or not removal of this tightly bound ADP affects the structure of the ncd motor domain (*17*, *18*). Far UV CD spectra indicated that the overall secondary structure was not substantially altered, while some differences were detected in the near UV CD spectra. Further investigation of the latter suggested that the adenine moiety of ADP was mainly responsible for the differences. This conclusion was supported by the difference UV absorption spectra (*18*). Changes in the CD spectrum of adenine nucleotide upon binding to enzymes have been reported, as far as we know, only once for creatine kinase (*19*), suggesting that ncd is very special as an adenine nucleotide-binding enzyme.

The CD bands are, in general, brought about by the interactions of transition-moment-dipoles between chromophores, whereas fluorescence reflects the environment, related to the local structure in many cases, of a fluorophore. The tryptophan fluorescence of the ncd motor domain is also affected by removal of the tightly bound ADP (*18*). The ncd motor domain has only two tryptophanyl residues, Trp370 and Trp473 (*10*). Trp370 is closer to, though not in direct contact with, the bound ADP than Trp473, which is almost at the opposite end of the ncd motor domain. We speculated that Trp370 would be responsible for the difference in fluorescence. In this study, we constructed ncd motor domain mutants by replacing one of the two tryptophanyl residues with a tyrosyl residue. We were also interested in the mutant ncd motor domain whose tyrosyl residue (Tyr442) in stack with the adenine moiety of ADP was replaced with a phenylalanyl residue. Here, we report on the spectroscopic characteristics of these mutant ncd motor domains, though in the presence of 0.5 M NaCl, and discuss structural implications based on these analyses.

MATERIALS AND METHODS

Chemicals—Commercially obtained ATP was used after purification by DEAE-Sephadex chromatography as described (*20*). MilliQ water was used throughout. The reagents were analytical grade.

Preparation of Tubulin—Tubulin was prepared from porcine brains as described previously (*21*). The separation of tubulin from MAPs (microtubule-associated proteins) was performed by DEAE-Sephacel chromatography, and the MAP-free tubulin was frozen in liquid nitrogen and stored at –80°C until use. Microtubules were polymerized by thawing this frozen tubulin solution

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at room temperature and by adding paclitaxel to a final concentration of 20 μM. After 20 min at room temperature, the polymerized microtubules were pelleted by centrifugation through a 10% sucrose cushion in 0.1 M MOPS-NaOH (pH 7.0), 2 mM $MgCl_2$, 0.5 mM EGTA, and 5 μM paclitaxel, and then resuspended in the above solution without sucrose.

Preparation of ncd Motor Domain and Its Mutant—The ncd motor domains, with mutations in Trp370, Trp473, or Tyr442 were constructed as follows. The ncd motor domain (amino acids 335–700) was PCR-amplified from pBS-NCD (a kind gift from Prof. L.S.B. Goldstein, University of California, San Diego; see Ref. *22*), which contained the entire coding sequence for ncd, and then cloned into the pET17b expression vector (Novagen, Madison, WI, USA). Site-directed mutagenesis was performed with a Mutan-K kit (Takara, Tokyo, Japan) or a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA).

Preparation of the wild type (WT) or mutant ncd motor domain protein was performed in the same way as described previously (*7*): cell lysates of *E. coli* that expressed the protein were applied to an SP-Sepharose column equilibrated with a solution consisting of 20 mM MOPS-NaOH (pH 7.0), 0.1 M NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.2 mM DTT and 0.02 mM ATP, and the ncd motor domains were eluted with a NaCl gradient. The fractions containing ncd motor domain were collected, dialyzed against the solution described above, and then applied to a small Q-Sepharose column. The flow-through fractions were pooled, supplemented with 0.5 mM ATP, and then rechromatographed on a small SP-Sepharose column in the same manner as above. ATP and sucrose were added to the resultant purified protein solution to 0.02 mM and 5%, respectively, and the solution was frozen in liquid nitrogen and stored at –80°C until use. The molecular mass and mean residue weight of the ncd motor domain were taken to be 41,300 and 113.2, respectively. These values were also used for the mutant proteins. The protein concentration was estimated by the Bradford method, which was calibrated by the method of Lowry *et al.* (*23*), using bovine serum albumin as a standard.

Preparation of ADP-Free ncd Motor Domain Mutants—To remove the tightly bound ADP, the WT or mutant ncd motor domain solution was brought to 0.5 M NaCl and 4 mM EDTA and passed through a small prepacked gel filtration column (10DG from Bio-Rad, Hercules, CA, USA) equilibrated with a solution consisting of 20 mM MOPS-NaOH (pH 7.0), 0.5 M NaCl, 2 mM EDTA and 0.2 mM DTT, as previously described (*17*, *18*). The pooled fractions containing the ADP-free WT or mutant ncd motor domain were brought to 4 mM $MgCl_2$ immediately after preparation.

In the case of the WT protein before EDTA-NaCl treatment, upon the addition of perchloric acid to 0.3 M to precipitate the protein, a substance was released into the supernatant whose UV absorption spectrum was identical to that of ADP (*17*, *18*). Likewise, the mutant proteins released ADP, and we estimated, based on the absorbance at 260 nm, that about 50 to 70% of the mutant proteins retained bound ADP. This percentage was lower than that in the case of the WT protein (*ca.* 80%, see Ref. *18*), possibly reflecting the less stable nature of the mutants or weaker ADP binding, even though ATP was present during the SP-Sepharose chromatography. On the other hand, when the WT or mutant proteins were gel-filtrated in the presence of EDTA and high salt as described above and subjected to perchloric acid precipitation, little UV-absorbing material was released, indicating that these proteins treated with EDTA and high salt were essentially free of bound ADP (*17*).

The ADP-free WT ncd motor domain was previously shown to bind a fluorescent ATP analog rather slowly (0.07 s^{-1}, but much faster than the basal ATPase rate, 0.003 s^{-1}; also see Ref. *17*). Our preliminary results showed that all the ADP-free forms of mutants also bound a fluorescent ATP analog slowly, just like the corresponding WT protein.

It is important to note that with W473Y, the ADP-free protein started precipitating ca 20 min after a shift of the temperature to 20°C, even in the presence of high salt, while no such precipitation was observed with the WT protein, W370Y or Y442F. The ADP-free W473Y was soluble at least for 4 h at 5°C and thus the measurements with W473Y were performed at this temperature, while they were carried out at 20°C for the other mutants. Within the time range of our CD or fluorescence measurements (within 2.5 h after preparation of the ADP-free forms that were kept at 5°C), the spectra did not change, suggesting these proteins remained stable for at least 2.5 h. It should also be noted that with the WT protein, the CD spectra were not affected on shifting of the temperature from 20 to 5°C (data not shown).

Spectroscopic Measurements—On the day of spectroscopic measurements, to prepare ADP-bound forms of the WT or mutant ncd motor domain, the protein solution was thawed and passed through a 10 DG gel filtration column equilibrated with a solution consisting of 20 mM MOPS-NaOH (pH 7.0), 0.5 M NaCl, 4 mM $MgCl_2$, 2 mM EDTA and 0.2 mM DTT. The ADP-free forms were prepared as described above just prior to the measurements. The final solution ingredients for ADP-free forms and for the forms with bound ADP were exactly the same.

The CD spectra were measured with a Jasco J-805 spectropolarimeter with a temperature-controlled cuvette holder as described previously (*17*, *24*). Fluorescence spectra were obtained with a Shimadzu RF-5000 spectrofluorometer. Measurements were carried out at 20 or 5°C as described above.

ATPase Activity Assay—The ATPase activity was measured as described (*7*). The reaction mixture comprised 0.1 M MOPS-NaOH (pH 7.0), 2 mM $MgCl_2$, 0.5 mM EGTA and 0.5 mM ATP. The enzyme reaction was allowed to proceed at 25°C and terminated by the addition of a final 0.3 M perchloric acid. Inorganic phosphate liberated on the ATPase reaction was determined by the modified malachite green assay (*25*) to calculate the enzyme activity.

RESULTS

Preparation of Mutant ncd Motor Domains—The preparation of mutant proteins was attempted from cell lysates of *E. coli* transformants. We were able to obtain soluble mutant ncd motor domains, W370Y and W473Y (tryptophanyl residues replaced with tyrosyl residues),

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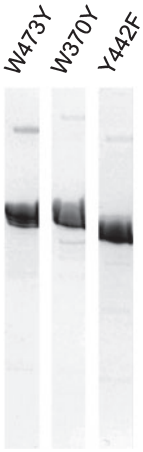

Fig. 2. **The mutant proteins.** SDS polyacrylamide gel electrophoresis patterns show the purified ncd motor domain mutants used for the spectroscopic and ATPase experiments. The molecular weights of the mutant proteins were essentially the same (the mobility on the gels differed to some extent because different types of gels were used on different days).

although the yields were lower than with the WT version (Fig. 2). In contrast, we did not recover any soluble protein when the tryptophanyl residues were replaced with alanyl ones (W370A or W473A).

As for the tyrosyl residue (Tyr442) in stack with the adenine moiety of ADP, we constructed mutants by replacing this residue with a phenylalanyl, histidyl, alanyl, or tryptophanyl residue. Only the phenylalanyl replacement (Y442F) resulted in some soluble protein in a yield comparable to that of W370Y or W473Y (Fig. 2). We failed to obtain other Tyr442 mutants in soluble forms. This side chain may be critical for the protein to make proper contacts with ADP in a stack, and replacement of this tyrosyl residue with another amino acid residue may be detrimental to the overall structure of the protein.

Table 1. **ATPase activity of the WT and mutant ncd motor domains.**

Protein	Basal activity	Microtubule activation	
		V_{max}	MT conc. for half max. activation
WT	0.0030 s^{-1}	2.3 s^{-1}	3.0 μM
W370Y	0.0022	0.83	3.8
W473Y	0.0016	2.0	5.4
Y442F	0.0030	2.7	3.7

ATPase activity was measured as described under Materials and Methods (also, see Ref. 7). Basal activity means the steady state ATPase rate in the absence of microtubules. The parameters for microtubule activation were estimated by fitting the data to a hyperbola.

We investigated the fluorescence and CD spectra of the three soluble mutant motor domains, W370Y, W473Y and Y442F, in the presence of high salt as follows. The ADP-free forms of the WT and mutant proteins were prepared as described under "MATERIALS AND METHODS" (*17*, *18*).

ATPase Activities of Mutant ncd Motor Domains—The basal ATPase activity and microtubule-stimulated ATPase activity of the mutant proteins were measured with low salt, as shown in Table 1. As a kinesin family ATPase, the ncd motor domain exhibits a very low basal ATPase activity, but it is stimulated dramatically by microtubules (*7*). With W473Y and Y442F, the microtubule activation was similar to that of the WT protein (Table 1): that is, half maximal activation was seen at similar concentrations of microtubules and the maximal activity at infinite concentrations of microtubules was also in a similar range. Although the maximal activity of W370Y was less than half of that of the WT protein, probably due to the close proximity of the mutation to the ATPase site, we should be able to conclude that the mutant proteins retained the original or similar enzymatic characteristics.

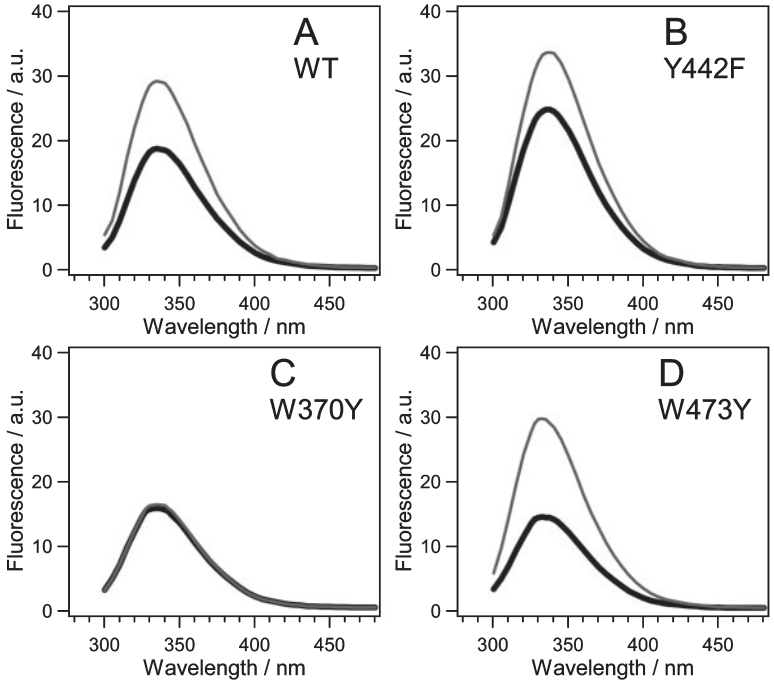

Fig. 3. **Fluorescence emission spectra of the WT and mutant ncd motor domains with excitation at 295 nm.** Fluorescence spectra of the ncd motor domain or a mutant were obtained as described under "MATERIALS AND METHODS." The temperature was controlled at 20°C by circulating water through the cuvette holder, while it was 5°C for W473Y. The protein concentration was 20 μM. Excitation was performed at 295 nm so that only tryptophanyl residues emitted fluorescence. A, the WT ncd motor domain: B, Y442F: C, W370Y: D, W473Y. The darker lines are the spectra of ADP-bound forms, the lighter ones being those of ADP-free ones.

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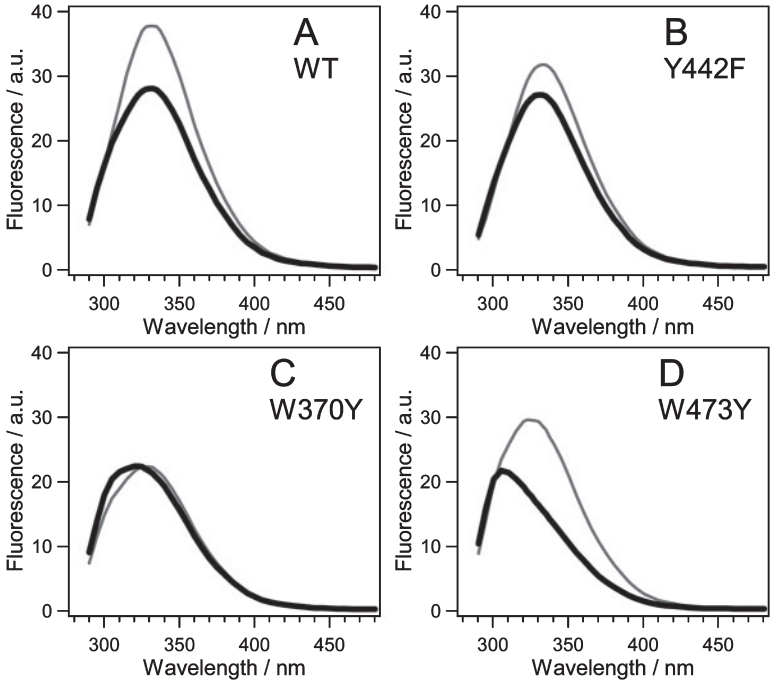

Fig. 4. **Fluorescence emission spectra of the WT and mutant ncd motor domains with excitation at 275 nm.** Fluorescence spectra of the ncd motor domain or a mutant were obtained in the same manner as described in the legend to Fig. 3, except that the excitation was performed at 275 nm so that both tryptophanyl and tyrosyl residues emitted fluorescence. A, the WT ncd motor domain: B, Y442F: C, W370Y: D, W473Y. The darker lines are the spectra of ADP-bound forms, the lighter ones being those of ADP-free ones.

Fluorescence Characteristics—As for the fluorescence emission spectra upon excitation of tryptophanyl residues at 295 nm, the ADP-free form of the WT ncd motor domain emitted more fluorescence than the ADP-bound form (Fig. 3A; also see Ref. *18*). Both W370Y and W473Y emitted fluorescence from the single tryptophanyl residue with the peak at 335 nm irrespective of the presence or absence of bound ADP, indicating that the environments of these two tryptophanyl groups are similar in terms of hydrophobicity. With W370Y, the spectra of the ADP-free and ADP-bound forms were almost completely superimposable, whereas with W473Y, the difference between the two forms was much bigger than that in the case of the WT protein (Fig. 3, C and D). These observations indicate that the structural environment of Trp473 is not influenced significantly by bound ADP, while that of Trp370 is affected to a considerable extent. These experiments demonstrate that the difference between the ADP-free and ADP-bound forms seen for the WT protein is due to Trp370, and that the contribution of Trp473 to this difference is negligible.

The spectra of Y442F were somewhat different from those of the WT protein in terms of the fluorescence intensity (Fig. 3B). It is suggested that the mutation of Tyr442 to phenylalanine has structural effects on the Trp370 environment, though the nature of the effects is unclear.

We also measured fluorescence emission spectra with excitation at 275 nm, where both tryptophans and tyrosines emit fluorescence, although the tryptophans are the major source as to fluorescence intensity. Indeed, the emission maximum was 332 nm in the presence and absence of the bound ADP for the WT ncd motor domain (Fig. 4A). Figure 4B shows the patterns for Y442F. The spectral difference between the ADP-bound form and the ADP-free form of Y442F was smaller than that in the case of the WT protein**.** With W370Y, the fluorescence intensity of the ADP-bound form was essentially the same as that of the ADP-free form, although the spectral shapes were slightly different from each other (Fig. 4C). With W473Y, the patterns were very different. In particular, the spectrum of the ADP-bound form was quite asymmetric, as can be seen in Fig. 4D (emission maximum, 305 nm).

We next examined difference fluorescence spectra with excitation at 275 nm. The difference spectrum between the WT ncd motor domain with bound ADP and a mutant (either W370Y or W473Y) showed a peak and a trough around 340 nm and 300 nm, respectively, as shown in Fig. 5A. The peak is thought to be due to the replaced tryptophanyl residue and the trough due to the tyrosyl residue introduced. The intensity of both the peak and the trough in the difference spectrum is larger for W473Y than for W370Y, which suggests that there are structural constraints placed on amino acid residue 473 that are stronger than those placed on residue 370. A major part of the structural constraints is likely due to the stacking of Trp473 and Tyr475 (see below). The difference spectrum between Y442F and the WT protein (Fig. 5A) shows a peak around 300 nm, probably due to the mutated tyrosyl residue. This suggests that there are no significant structural differences between the two in the presence of bound ADP.

The difference spectra between the ADP-bound and ADP-free forms are shown in Fig. 5B. W473Y has a single tryptophan at 370 and a peculiar difference spectrum with a peak at 332 nm was observed. This implies that the removal of ADP causes a structural change around residue 370, probably including reorientation of Trp370. In contrast, with W370Y, we saw a small peak around 340 nm and a trough around 300 nm. This suggests that the structural change around Trp473 upon removal of ADP is insignificant, and indicates that the fluorescence from the newly introduced Tyr370 and/or from Tyr372

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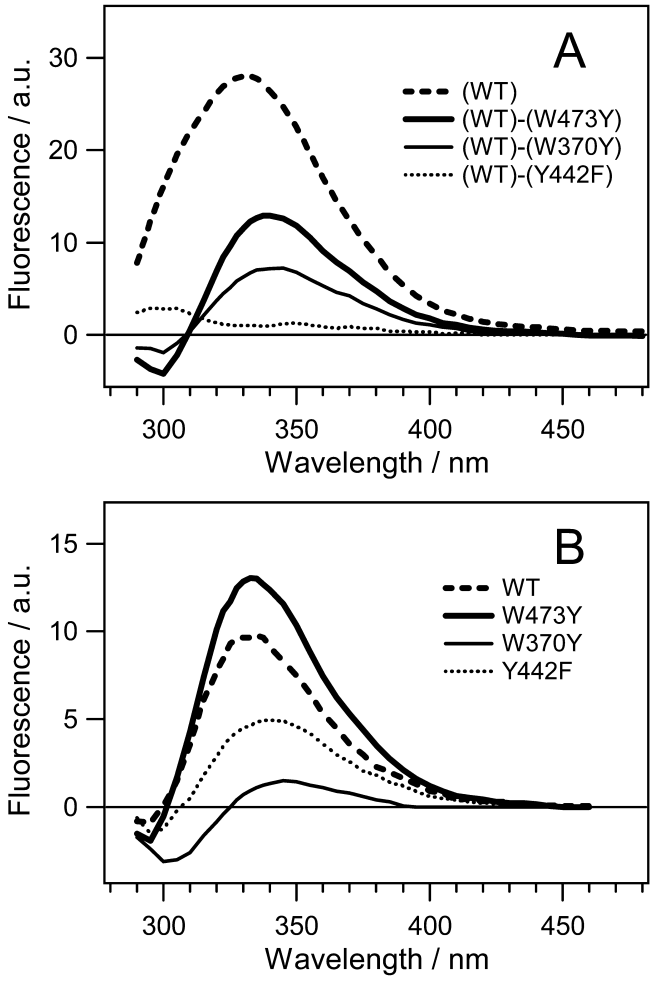

Fig. 5. **Difference fluorescence spectra of the ncd motor domain and mutant proteins with excitation at 275 nm.** The difference fluorescence spectra of the ncd motor domain and mutant proteins were calculated from those shown in Fig. 4. A shows the difference spectra between the WT protein and a mutant one, both with bound ADP. The broken line shows the fluorescence spectrum of the WT protein (not a difference spectrum). Bold line, W473Y subtracted from WT; solid line, W370Y subtracted from WT; dotted line, Y442F subtracted from WT. B shows the difference spectra between the ADP-free form and ADP-bound form (ADP-free minus ADP-bound): broken line, WT: bold line, W473Y; solid line, W370Y; dotted line, Y442F.

decreased. This decrease in tyrosine fluorescence suggests that the structural changes due to ADP removal weaken the constraints around newly introduced Tyr370, or that Tyr370 moves closer to amino acid residues that quench fluorescence. With Y442F, the difference spectrum is in between the two described above, namely those of W370Y and W473Y, making us suspect that, upon removal of the bound ADP, the fluorescence intensity of Tyr372 decreases while that of Trp370 increases.

CD Spectra of These Mutant ncd Motor Domains—The far UV CD spectra of the WT and three mutant proteins, W370Y, W473Y and Y442F, with bound ADP are shown in Fig. 6A. They were similar to one another, suggesting that the mutations did not affect the secondary structure to a significant extent. A minor difference was nevertheless revealed by difference spectra (Fig. 6B). WT versus W370Y and WT versus Y442F were almost flat, but WT *versus* W473Y showed a shallow trough around 225 nm. This trough is thought to be due to the tryptophanyl residue because of the trough's wavelength, which means that Trp473 gives a positive CD band in this range reflecting its tertiary structure. This likely originates from the transition-moment-dipole interaction of Trp473 and Tyr475.

The far UV CD spectra of the ADP-free forms of the WT and three mutant proteins are shown in Fig. 6C. The difference spectra between the ADP-free forms and ADP-bound forms of the ncd motor domains were superimposable, with a trough around 200 nm (Fig. 6D). This suggests that in each case the content of random coils decreased slightly upon removal of the bound ADP. It should be noted that on the structural analyses of the motor domain of KIF1A, Nitta *et al.* (*13*) observed considerable changes in the length of helix α4 depending on the nucleotide state.

Next, near UV CD spectra were investigated (Fig. 7A). As reported previously (*18*), we observed a conspicuous negative band at 255–265 nm that we ascribe to the transition-moment-dipole interaction between the bound ADP and Tyr442 stacked with it, as well as to that between the bound ADP and tryptophanyl residue(s). The signal intensity was large in the cases of WT and W473Y, while it was much less in the case of W370Y. Because the amount of bound ADP at the ATPase site did not differ to a considerable extent among the mutant ncd motor domains (still less than that of the WT protein), the difference between W473Y and W370Y should be meaningful. Thus, this strongly suggests that the tryptophan involved in the transition-moment-dipole interaction with the bound ADP was Trp370 but not Trp473. With Y442F, the negative CD at 255–265 nm was small, being comparable to that of W370Y. Since the transition-moment-dipole of phenylalanine is smaller than that of tyrosine, the interaction between Phe442 and the bound ADP would also be smaller.

We saw a positive CD band and a negative one at 285 nm and 291 nm, respectively, in the cases of the WT protein and Y442F, though the two bands overlapped each other, resulting in an apparent peak shift. Since the intensities of these bands changed upon removal of the bound ADP (Fig. 7B), we interpret these CD bands as being related to the interactions of transition-moment-dipoles of tryptophan and/or tyrosine(s) with bound ADP. W473Y was unique in that it did not exhibit the CD band at 291 nm, suggesting that this CD band is related to the stacking structure of Trp473 with Tyr475.

The ADP-free forms gave negative CD bands at both 285 nm and 291 nm (Fig. 7B), contrary to the WT and mutant proteins with bound ADP. This raises the possibility that Trp370 forms a new stacking structure with the proximal tyrosine residue following the removal of the bound ADP.

DISCUSSION

Previously, we reported that the tightly bound ADP of ncd motor domain could be removed by EDTA treatment only in the presence of high salt (0.5 M NaCl), but that

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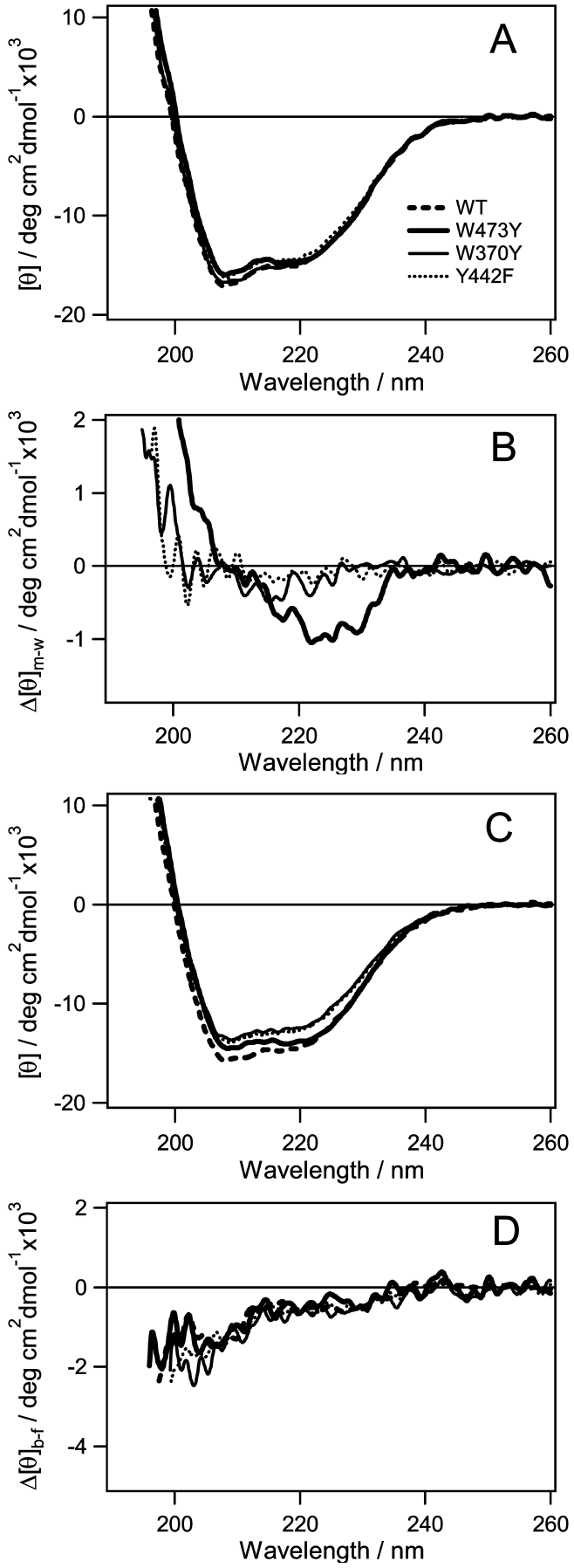

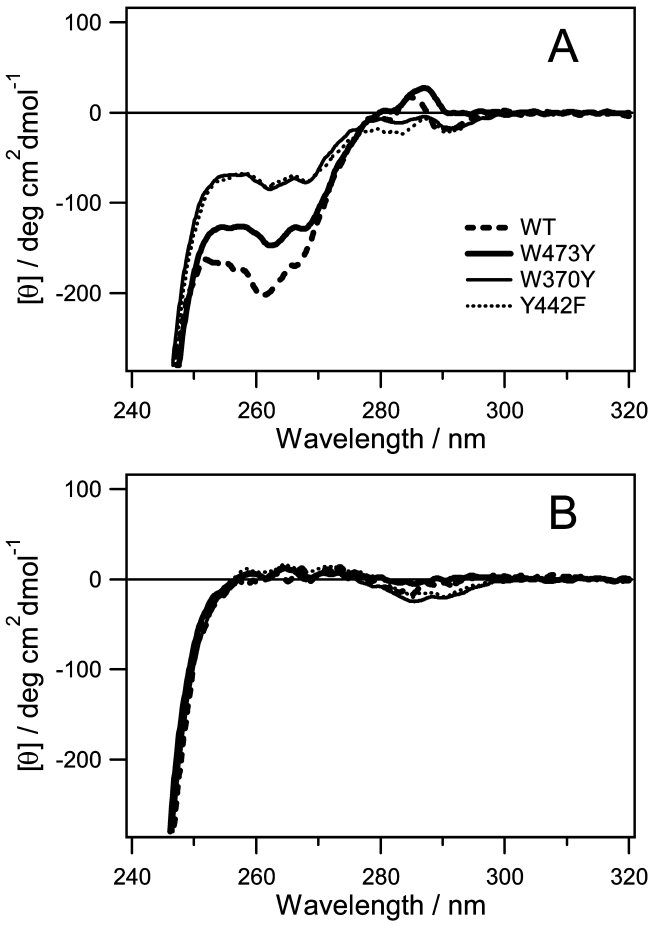

Fig. 7. **Near UV CD spectra of the ncd motor domain and mutant proteins.** The conditions for measurements were the same as given in the legend to Fig. 6, except that the light path was 10 mm. A indicates the spectra of the various ncd motor domain proteins with the bound ADP: WT, W473Y, W370Y or Y442F, shown by broken, bold, solid, or dotted line, respectively. B shows the spectra of the ADP-free forms of the WT and mutants, where the line definition is the same for each protein as in A.

EDTA treatment in the presence of 0.1 M NaCl denatured the protein instantaneously (*17*, *18*). Under this condition with high salt, however, we showed that the adenine moiety of tightly bound ADP of ncd motor domain gives a conspicuous CD band in the range of 255–265 nm. In general, CD bands are due to interactions of transition-moment-dipoles between chromophores. The

Fig. 6. **Far UV CD spectra of the ncd motor domain and mutant proteins.** The far UV CD spectra of the ncd motor domain and mutant proteins were obtained at 20°C except for W473Y (at 5°C). The cuvette light path was 0.2 mm for far UV CD spectra. The molar ellipticity, [θ], was calculated on the basis of the amino acid residue concentration; the mean residue weight was taken as 113.2 for the WT ncd motor domain as well as for a mutant protein. A shows the spectra of the ADP-bound forms of the WT and mutant proteins while C shows those of the ADP-free forms. For each illustration: broken line, WT; bold line, W473Y; solid line, W370Y; dotted line, Y442F. B shows the difference spectra, *i.e.*, spectrum of an ADP-bound mutant subtracted from that of the ADP-bound WT. D shows the difference spectra between the ADP-free form shown in C and the ADP-bound form shown in A (ADP-bound form minus ADP-free form), where the definition of the lines is the same as above.

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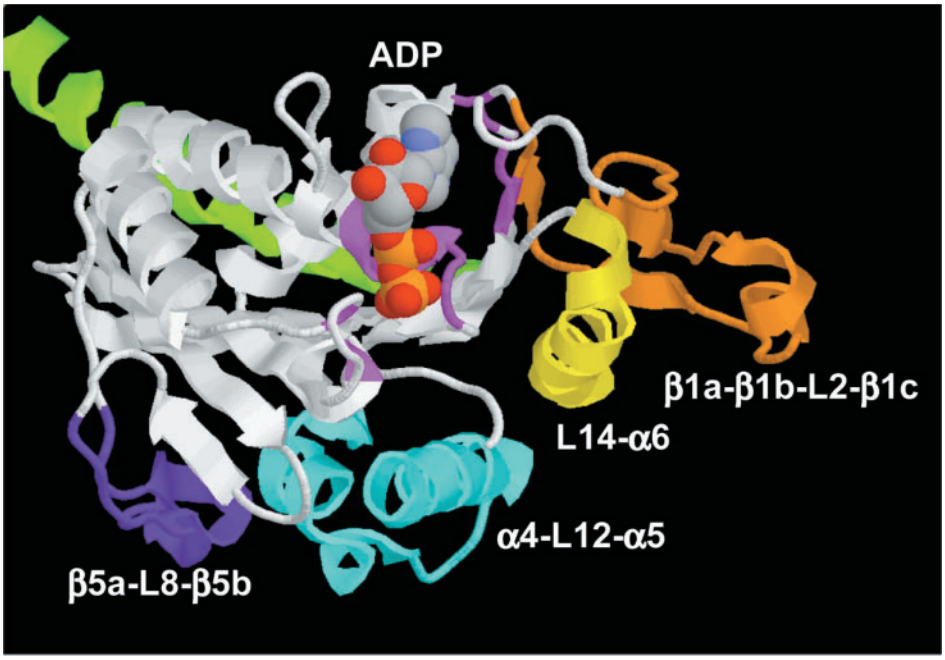

Fig. 8. **The regions of the ncd motor domain relevant to the observations and analyses in this study.** The two sites likely involved in the microtubule-binding (*27*) are shown in purple (β-5a-L8-β5b) and cyan (α4-L12-α5). Two regions forming the same plane as the core of the motor domain, the six-stranded β-sheet, are shown in yellow (L14-α6) and orange (β1a-L2-β1b-β1c). The ADP-binding site is shown in reddish purple, where ADP itself is depicted as a space-filling model.

candidate amino acid residues responsible for the interactions were Tyr442, which is in direct stack with adenine, and Trp370.

We also observed that the tryptophan fluorescence increased upon removal of the tightly bound ADP (*18*), which suggested a structural change in the environment of tryptophan(s). The ncd motor domain has only two tryptophanyl residues, Trp370 and Trp473 (*10*). Because Trp473 is much more distant from ADP than Trp370, we speculated that Trp370 would be responsible for the fluorescence change. In the previous study, however, we did not obtain specific evidence of this.

In order to clarify these points, we constructed ncd motor domain mutants: W370Y, W473Y and Y442F. Unfortunately, we cannot ascertain whether or not these mutants exhibit motility, since it is only possible to demonstrate motility through in vitro assays when ncd constructs include the coiled-coil stalk region, but not with the ncd motor domain alone, as used herein. Nevertheless, the mutants appeared to retain the original ATPase characteristics of the ncd motor domain, as can be seen in Table 1, though the kinetic parameters varied to some extent.

The near UV CD measurements suggested that the peculiar CD spectra observed for the WT protein were due to the interactions of adenine with Tyr442 and Trp370. Whereas the aromatic side chain of Tyr442 stacks directly with adenine, Trp370 is about 1.5 nm distant from the bound adenine. Nonetheless, this distance is within the range of transition-moment-dipole interactions (*18*). As can be seen in Fig. 7A, the spectra of Y442F and W370Y were similar to each other, suggesting that the extents of their influence on this characteristic circular dichroism were comparable. On the other hand, it is reasonable that Trp473 does not exhibit significant interaction with adenine, because it resides 3.8 nm away from adenine separated by the six-stranded β-sheet core.

As for the fluorescence change upon removal of the tightly bound ADP, we found that Trp370 was mainly responsible and that Trp473 made little, if any, contribution. The peak wavelength of fluorescence is indicative of the hydrophobicity of the environment in which a fluorophore resides. In our case, the peak wavelength did not change upon ADP removal, suggesting that the hydrophobicity did not change. On the other hand, the intensity change is indicative of structural changes. Our results suggest that the removal of the tightly bound ADP induces certain structural changes in the hydrophobic region including Trp370 (β1a in Fig. 8), even though Trp370 is not in direct contact with the region having the ADP-binding pocket (see below). In contrast, the structural change induced by the removal of ADP did not seem to be propagated to the region containing Trp473. Thus, removal of ADP induces measurable structural change(s) only in some specific region(s). This would not, however, exclude the possibility that during the mechanochemical ATPase cycle, more dynamic changes would take place and that even the region including Trp473 would undergo some structural changes (*4*, *26*).

Recently, Nitta *et al.* (*13*) studied the three-dimensional structures of the KIF1A motor domain with various ATP analogs bound at the ATPase pocket. They detected many important changes in the structure depending on the nucleotide states, one of which was the positions of L9 of Switch I and of L11 of Switch II. They speculated that bringing these loops to microtubules might open the nucleotide-binding pocket and, consequently, might make the binding of nucleotide considerably weak. Our observations in this study and the previous ones might reflect a similar state of the motor domain, although our studies did not focus on L9 or L11. In addition, we have to note that our observations were made in the presence of high salt (0.5 M NaCl). Nonetheless, it will be interesting to interpret our current results when the three-dimensional structure of the nucleotide-

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free motor domain of kinesin or one of its family members is solved in the future.

Our detailed analyses of fluorescence and circular dichroism also revealed certain local structural features of the ncd motor domain, as described under Results. One is the stacking of Trp473 and Tyr475 suggested by the difference fluorescence spectra (Fig. 5A) and by the CD spectral analyses (Figs. 6B and 7). This is consistent with that mutation of Trp473 to tyrosine resulted in thermal instability of W473Y, which also reminds us that Trp473 is well conserved among C-terminal kinesin motors. The other, suggested by CD spectral analyses, is that the removal of ADP may allow Trp370 to form a new stacking structure with the proximal tyrosyl residue, which may be one of the reasons for enhancement of the fluorescence from Trp370. These results obtained with the CD measurements are in good accord with structural change(s) expected from the fluorescence measurements.

Spectroscopic studies such as our current one could detect some local structural features of proteins of concern, but unlike X-ray crystallography, spectroscopy is an indirect tool with certain limitations. Nonetheless, our studies should supplement previous X-ray crystallographic investigations. Since the motor domain of ncd has an aromatic amino acid residue stacked with adenine of ADP, and since it has only two tryptophanyl residues, our previous and current studies may be of more advantage in comparison with other studies on proteins in general. In addition, we should note once again that the ADP-free form of ncd motor domain or one of its mutants has not been crystallized yet, where spectroscopy has some roles to play.

From the alanine-scanning experiments on the kinesin motor domain by Woehlke *et al*. (*27*) and from the cryo-electron microscopic observations of the microtubule-ncd complex (*28*, *29*; also see Ref. *30*), the regions involved in microtubule-binding are thought to be L8 and L12-α5 (depicted in Fig. 8), as well as L7 and L11. The binding of the ncd or kinesin motor domain to microtubules weakens the ADP-binding (*6*, *7*), probably influencing the structure of the nucleotide-binding pocket. Likewise, ATP-binding causes dissociation of the ncd or kinesin motor domain of the microtubule-motor domain complex (*6*, *13*, *31*). During the mechanochemical ATPase cycle, it is likely that the ncd motor domain undergoes dynamic structural changes (*4*, *6*, *32*, *33*). We plan to introduce appropriate cysteine residues, for example, to the microtubule-binding regions or in the vicinity of Trp473, which can be modified chemically with fluorescent reporters for additional structural studies of the mechanism of kinesin-based motility (*32*).

We wish to thank Prof. Dan Kiehart (Duke Univ.) for critical reading of the manuscript and many valuable comments, and Ms. Takako Seki for her skillful technical assistance.

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