Hydrolysis of AMPPNP by the motor domain of ncd, a kinesin-related protein

Yoshikazu Suzuki[a], Takashi Shimizu[b,c,*], Hisayuki Morii[b], Masaru Tanokura[a]

[a]*Biotechnology Research Center, University of Tokyo, Bunkyo, 113 Tokyo, Japan*
[b]*National Institute of Bioscience and Human-Technology, Higashi, Tsukuba, 305 Ibaraqi, Japan*
[c]*National Institute for Advanced Interdisciplinary Research, Higashi, Tsukuba, 305 Ibaraqi, Japan*

Received 3 March 1997

Abstract AMPPNP was found to be hydrolyzed by the motor domain of ncd (the product of a *Drosophila* gene, *non-claret disjunctional*), a kinesin-related protein. This hydrolysis could be monitored by ^{31}P NMR spectroscopy and by an assay of phosphate, one of the products of the hydrolysis. The rate was ≈ 0.00004 s^{-1}, 1% of the ATP turnover rate. The AMPPNP turnover was not stimulated by microtubules. Kinesin motor domain also turned over AMPPNP but at a somewhat lower rate. Although the turnover was slow, the present finding may present an important caveat, since AMPPNP has been widely used for investigations of kinesin and kinesin-related proteins as a non-hydrolyzable ATP analogue.

Key words: Adenylylimidodiphosphate; Ncd; Kinesin; Molecular motor; ^{31}P NMR

1. Introduction

ncd, the product of the *non-claret disjunctional* gene of *Drosophila*, is a 700-amino-acid residue protein, having a C-terminal globular domain that is homologous to the motor domain of kinesin, a microtubule motor protein [1–3]. The motor domain of kinesin or a kinesin-related protein such as ncd has an ATPase site and an ATP-dependent microtubule-binding site, and is thought to generate movements along microtubules. Recently, the 3-dimensional structures of kinesin and ncd motor domains have been determined by X-ray crystallography [4,5]. In spite of the fact that the motility directionality of ncd along a microtubule (i.e., minus-end-directed) is opposite to that of kinesin (plus-end-directed), those studies revealed that the structures of the motor domains are remarkably similar to each other.

One characteristic feature of kinesin is the tight binding to microtubules in the presence of AMPPNP, an ATP analogue. This property is shared by all the kinesin superfamily members examined so far [6–8]. In fact, the original kinesin purification protocol utilized this characteristic; microtubule affinity in the presence of AMPPNP [9,10]. In spite of this remarkable effect of AMPPNP on kinesin superfamily members, the interaction of kinesin or a kinesin-related protein with AMPPNP has not been studied in detail so far, except for a recent investigation on the equilibrium dissociation constant of mant-AMPPNP to kinesin motor domain [11].

Here, we present data that indicate the hydrolysis of AMPPNP, which is widely used as a non-hydrolyzable ATP analogue, by the motor domain of ncd and by that of kinesin, although the turnover was slow. In addition, we report that the turnover was not likely to be accelerated by microtubules, suggesting that the rate-limiting step of AMPPNP turnover should be different from the ADP release step which is likely to be the rate-limiting step of ATP turnover. On the other hand, we could not detect a sign of AMPPCP hydrolysis by ncd or kinesin motor domain.

2. Materials and methods

2.1. Chemicals

Taxol was a generous gift by Dr. M. Suffnes, National Cancer Institute, USA. ATP, AMPPN, AMPPNP and AMPPCP (Boehringer) were purified by DEAE-Sephadex chromatography with a triethylammonium bicarbonate buffer (pH 7.6) gradient, and converted into sodium salt by passing through a small Dowex 50W (H^+) column and by titrating with NaOH. It should be noted that, even after purification, AMPPNP contained small amounts of hydrolysis products (Fig. 1a). [γ-^{32}P]ATP (PB170) was from Amersham. Other reagents were of analytical grade.

2.2. Preparation of proteins

The ncd motor domain (*Drosophila* ncd construct containing residues 335–700) [4] and K349 [5], a kinesin motor domain (human kinesin heavy-chain construct containing residues 1–349), were expressed in *E. coli* and purified as described previously [12,13]. Both are monomeric, and the molecular mass of ncd motor domain was taken to be 41.3 kDa and that of K349 to be 39.2 kDa. Tubulin was prepared from porcine brain supernatant by the method described in [14].

2.3. Assays

The assay method for ATP or AMPPNP turnover is described in the figure legend or in Table 1.

The protein concentration was measured by the method of Lowry et al. [15], using bovine serum albumin as a standard.

2.4. Spectroscopic measurements

^{31}P NMR measurements were performed on a JOEL α500 spectrometer at 202 MHz and 20°C, the details of which will be described elsewhere [Suzuki, Tanokura and Shimizu, unpublished]. For CD measurements, a Jasco J-600 CD spectropolarimeter was used with a temperature controlled cuvette-holder at 20°C [16]. Prior to the CD measurements, the protein solutions were clarified by centrifugation at 14 000×*g* for 10 min, and turbidity development was not detected after the measurements.

*Corresponding author. National Institute of Bioscience and Human-Technology, Higashi, Tsukuba, 305 Ibaraqi, Japan. Fax: (81) 298-54-6192

Abbreviations: AMPPCP, adenylylmethylenediphosphonate; AMPPN, adenosine 5′-diphosphoramidate; AMPPNP, adenylylimidodiphosphate; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; mant-ADP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-diphosphate; mant-AMPPNP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenylylimidodiphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid

PII S0014-5793(97)00472-9

3. Results

3.1. Hydrolysis of AMPPNP by ncd motor domain detected by ^{31}P NMR

The motor domain of ncd, like that of kinesin [5,17], contains tightly bound ADP at its catalytic site [4,12]. During a course of our study on the ^{31}P NMR spectroscopy of the tightly bound ADP of ncd motor domain [Suzuki et al., unpublished], we were also interested in looking at ^{31}P NMR spectra of ncd motor domain/AMPPNP complex. Since it was impossible to exchange the tightly bound ADP with AMPPNP by a simple addition of AMPPNP to ncd motor domain/ADP complex, we first removed the bound ADP by gel filtration in the presence of 0.5 M NaCl and 2 mM EDTA [16], and incubated this nucleotide-free ncd motor domain with AMPPNP. As seen in Fig. 1, we did not detect a signal due to the γ-phosphorus of AMPPNP bound to ncd motor domain; the signal might exhibit the same chemical shift as that of the γ-phosphorus of free AMPPNP; or it might be invisible due to some reasons. On the other hand, we observed the β-phosphorus signal (β′ in the figure) of AMPPNP (or AMPPN) bound to the ncd motor domain, the assignment of which will be described elsewhere [Suzuki et al., unpublished]. The α-phosphorus signal of the bound AMPPNP (or AMPPN) was not detected, the reason for which may be the same as that for the γ-phosphorus signal described above.

To our surprise, on the other hand, we detected increasing amounts of inorganic phosphate with incubation time and of new signals that were likely due to the α- and β-phosphorus of free AMPPN since their chemical shifts were the same as those of authentic AMPPN (data not shown), one of the hydrolysis products of AMPPNP. This observation indicated that AMPPNP is not an absolutely non-hydrolyzable analogue of ATP for an enzyme like ncd motor domain. Such hydrolysis of AMPPNP has, in fact, been observed with sarcoplasmic reticulum Ca-ATPase [M. Kawakita, personal communication].

3.2. AMPPNP turnover by ncd motor domain measured by phosphate assay and the effect of microtubules on the turnover

Next, we assayed the turnover rate of AMPPNP by ncd motor domain by phosphate determination. Since ncd motor domain contains tightly bound ADP which may act as an inhibitor for the AMPPNP turnover, we measured the rate by normal ncd motor domain (i.e. with bound ADP) and that by ncd motor domain free from bound ADP. In the presence of 0.5 M NaCl, both rates agreed within the range of experimental error as shown in Table 1: $\approx 0.00004\ s^{-1}$, about 1% of the ATP turnover rate under the same condition. We also observed that K349, a kinesin motor domain, could hydrolyze AMPPNP. The turnover rate was a little lower than that by ncd motor domain. On the other hand, we did not detect any appreciable turnover of AMPPCP by either ncd motor domain or K349. AMPPCP may be truly non-hydrolyzable for these motor domains, or at least the turnover would be extremely slow.

The ATP turnover by ncd motor domain or K349 is shown to be stimulated 1000-fold or more by microtubules [8,12,18]. This stimulation is considered to be due to the acceleration of the ADP release step, although some opposing observation

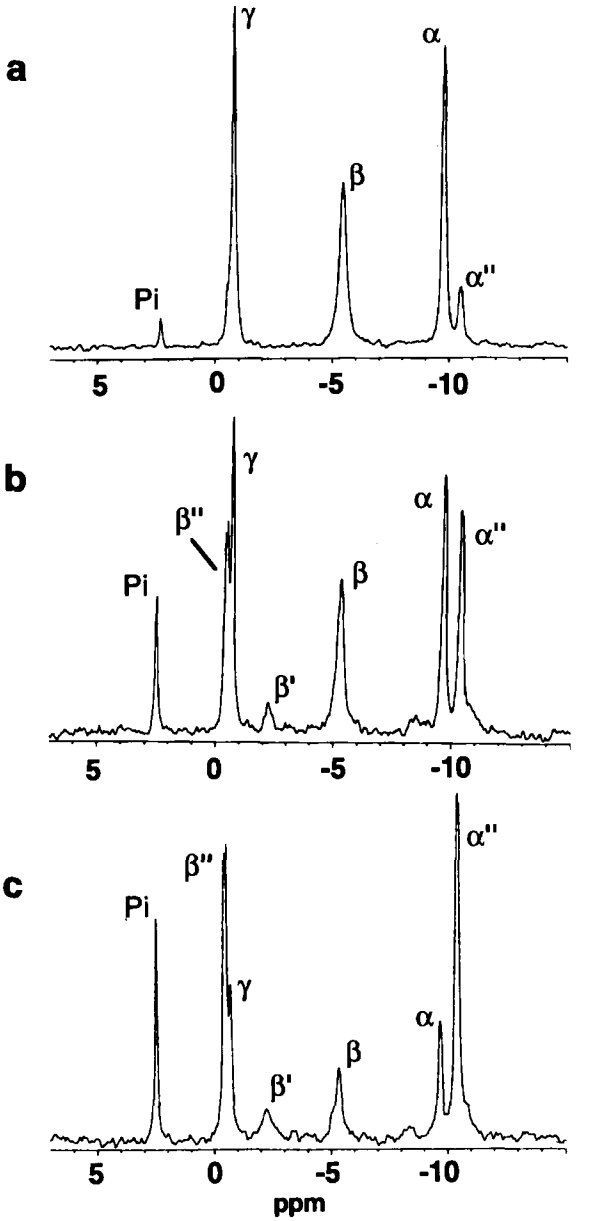

Fig. 1. ^{31}P NMR spectra of AMPPNP in the presence of ncd motor domain. ^{31}P NMR spectra were recorded as briefly described in Section 2. The nucleotide-free ncd motor domain (1.6 mM (66 mg/ml) final) in a 60% D_2O solution containing 20 mM Mops-NaOH (pH 7), 0.5 M NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA and 2 mM DTT was incubated with 3.2 mM AMPPNP at 20°C. NMR scans were accumulated for the first 70 min (b) or for 70 min from 210 to 280 min (c) after the beginning of the incubation. a: The spectrum accumulated for the first 70 min in the absence of the enzyme. α, β and γ denote the signals due to the corresponding phosphorus of free AMPPNP. Pi is the signal due to inorganic phosphate. β′ indicates the signal of β-phosphorus of AMPPNP (or AMPPN) bound to ncd motor domain; the assignment will be described elsewhere [Suzuki et al., unpublished]. α″ and β″ are the signals due to α- and β-phosphorus of free AMPPN identified by the use of authentic AMPPN. Note that the signals due to free AMPPNP decreased with time in the presence of ncd motor domain, while those due to inorganic phosphate and AMPPN increased. These changes were very slow, if any, in the absence of the enzyme. As noted in Section 2, AMPPNP contained small amounts of hydrolysis products even after purification.

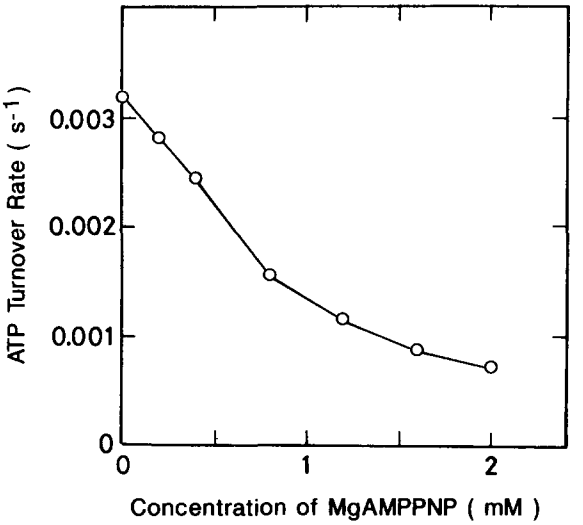

Fig. 2. Effect of AMPPNP on the ATP turnover by ncd motor domain. The ATP turnover was assayed at 20°C in the assay mixture consisting of 20 mM Mops-NaOH (pH 7.0), 0.5 M NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 μM [γ-^{32}P]ATP, 0.1 μM ncd motor domain, and 0 to 2 mM Mg-AMPPNP. After 10 min incubation, the enzyme reaction was terminated by adding 0.3 M perchloric acid, and then final 50 mg/ml activated charcoal and 5 mM inorganic phosphate were added. After centrifugation, an aliquot of the supernatant was transferred to a scintillation vial, and the radioactivity was determined by Cerenkov counting.

has also been reported [19]. The AMPPNP turnover by ncd motor domain was very moderately, if any, stimulated by microtubules (Table 1). Although a possibility such that microtubules of much higher concentration, i.e. 50 mg/ml, than used herein would stimulate the turnover could not be excluded, large stimulation would be unlikely to take place. This may suggest that the rate-limiting step of the AMPPNP turnover may be different from the ADP (as a matter of fact, AMPPN) release.

3.3. Inhibition by AMPPNP of ATP turnover by ncd motor domain

We also studied the AMPPNP-induced inhibition of the ATP turnover by ncd motor domain. As shown in Fig. 2, the inhibition was considerably weak; half maximal inhibition of ATP turnover at an ATP concentration of 1 μM was observed at 0.8 mM AMPPNP. This suggests that the apparent affinity of AMPPNP is likely to be more than 2 orders lower than ATP, although we must note that since we did not measure the binding constants of ATP or AMPPNP, the above-mentioned value is just a rough estimation.

3.4. CD spectra of ncd motor domain in the presence of AMPPNP

We previously reported that although the backbone structure of ncd motor domain or K349 was not likely to change by the removal of tightly bound ADP in the presence of high salt as revealed by far UV CD spectra, the near UV (240–300 nm) CD spectra differed depending on the nucleotide state, i.e. with or without bound ADP [16].

Here we investigated the CD spectra after addition of AMPPNP to the ADP-free ncd motor domain. The far UV CD spectrum was essentially the same as that of normal ncd motor domain (data, not shown). In the near UV region, nucleotide-free ncd motor domain gave a spectrum distinct from that of ordinary ncd motor domain (with bound ADP) (Fig. 3) as reported previously [16]. Addition of excess Mg^{2+} ion and ATP to nucleotide-free ncd motor domain changed the spectrum considerably (Fig. 3), and the extent of the change was similar to that observed after addition of Mg^{2+} ion and ADP. On the other hand, the near UV CD spectrum after the addition of 0.15 mM AMPPNP (and excess Mg^{2+} ion) was different from that of normal ncd motor domain as well as from that of the nucleotide-free ncd motor domain; in between the two (Fig. 3). Varying the AMPPNP concentration to 0.1 mM or to 0.2 mM did not significantly change the spectrum, so that it is unlikely that AMPPNP added was much less than saturating to give an intermediate pattern. Addition of 0.15 mM AMPPCP or AMPPN to nucleotide-free ncd motor domain in the presence of excess Mg^{2+} ion gave near UV CD spectra almost superimposable to that in the presence of AMPPNP (Fig. 3). One possible explanation for the intermediate pattern in the presence of AMPPNP may be that ncd motor domain/AMPPN(P) would take some different state from that of ncd motor domain/ADP complex.

4. Discussion

Herein we showed that ncd motor domain or kinesin motor domain (K349) could slowly hydrolyze AMPPNP, an ATP analogue widely used as non-hydrolyzable. In fact, AMPPNP could be cleaved by a certain enzyme as described above. Moreover, an investigation of AMPPNP binding to myosin S1 by ^{31}P NMR by Shriver and Sykes [20] seems to have indicated slow turnover of AMPPNP by this motor enzyme, although the authors did not mention it in their paper. In any event, these results including ours herein are a caveat in using

Table 1
Turnover of AMPPNP by ncd motor domain or by K349

Enzyme	Microtubule concentration	Turnover rate(s^{-1})
ncd motor domain	0	0.000042
ncd motor domain (nucleotide-free)	0	0.000037
ncd motor domain	1 mg/ml	0.000046
ncd motor domain	2 mg/ml	0.000038
K349	0	0.000016
K349 (nucleotide-free)	0	0.000023

Turnover of AMPPNP was assayed in an assay mixture containing 20 mM Mops-NaOH (pH 7), 0.5 M NaCl, 2 mM $MgCl_2$, and 10-20 μM one of the motor domains. Nucleotide-free ncd motor domain or K349 was prepared as described [16]. The incubation was done at 20°C for up to 2 h, and the reaction was terminated by adding 0.3 M perchloric acid. The phosphate liberated was determined by modified malachite green method [21,22]. In the case of the microtubule (taxol-stabilized) addition, the NaCl concentration was 0.1 M. It should be noted that the AMPPNP turnover rate by ncd motor domain in the presence of 0.1 M NaCl (and in the absence of microtubules) was comparable to that in the presence of 0.5 M NaCl.

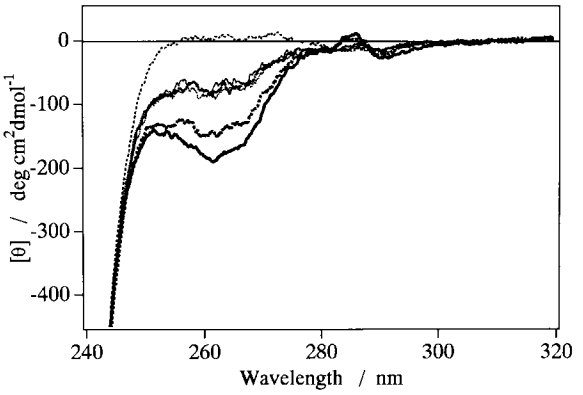

Fig. 3. Near UV CD spectra of ncd motor domain with various nucleotide conditions. ncd motor domain free from bound ADP was prepared, and this nucleotide-free ncd motor domain (0.05 mM) in a solution containing 20 mM Mops-NaOH (pH 7), 0.5 M NaCl, 2 mM EDTA and 0.5 mM DTT was incubated on ice for 2 h after the addition of 4 mM $MgCl_2$ with 0.15 mM ATP (bold, broken), AMPPNP (medium, dotted), AMPPCP (thin, solid) or AMPPN (thin, dotted). CD spectra were then recorded at 20°C using quartz cuvettes with 2 mm light path. The spectrum of original ncd motor domain, i.e., with bound ADP (bold, solid), and that of nucleotide-free ncd motor domain (medium, broken) are also shown. Note that the spectra with AMPPNP, AMPPCP or AMPPN were almost identical.

AMPPNP; in certain cases, it should be handled as a slowly hydrolyzable ATP analogue. On the other hand, AMPPCP, another analogue of ATP, was likely to be non-hydrolyzable for ncd motor domain or K349.

With ncd motor domain or kinesin motor domain, the ADP release step is thought to mainly contribute to the overall turnover rate not only in the absence of microtubules but in their presence [[6,8,12,18]; however, refer to [19]]. The present results suggest that in the case of the AMPPNP turnover, some other step should be rate-limiting. Most likely candidate may be the hydrolysis step, and the major intermediate may be the motor domain/AMPPNP complex.

Quite recently, Rosenfeld et al. [11] reported that the equilibrium dissociation constant of mant-AMPPNP, a fluorescent AMPPNP analogue, to kinesin motor domain was 7 μM, about 10-fold larger than that of mant-ADP, although they handled mant-AMPPNP as non-hydrolyzable. Our current study suggested that the binding was likely to be considerably weaker than that of ATP, in accordance with the results by Crevel et al. [7]. Although the binding of ADP to ncd or kinesin motor domain is tight, it should be weaker than that of ATP, and the binding of AMPPNP would be further weaker than that of ADP. Thus, our estimation may be consistent with the previous results [11].

Acknowledgements: We thank Drs. Jonathan Kull and Elena Sablin (University of California, San Francisco, CA) for providing the plasmid for K349 and that for ncd motor domain, respectively. We are also grateful to Dr. David Hackney (Carnegie Mellon University) for stimulating discussion. Technical assistance by Ms. Lisa Yamaguchi is acknowledged. This work was supported by Grants-in-Aid from the Agency of Industrial Science and Technology, MITI (T.S. and H.M.) and from the Ministry of Education, Science and Culture (Y.S. and M.T.).

References

[1] S.A. Endow, S. Henikoff, L. Soler-Niedziela, Nature 345 (1990) 81–83.
[2] H.B. McDonald, R.J. Stewart, L.S.B. Goldstein, Cell 63 (1990) 1159–1165.
[3] R. Chandra, E.D. Salmon, H.P. Erickson, A. Lockhart, S.A. Endow, J. Biol. Chem. 268 (1993) 9005–9013.
[4] E.P. Sablin, F.J. Kull, R. Cooke, R.D. Vale, R.J. Fletterick, Nature 380 (1996) 555–559.
[5] F.J. Kull, E.P. Sablin, R. Lau, R.J. Fletterick, R.D. Vale, Nature 380 (1996) 550–555.
[6] A. Lockhart, R.A. Cross, Biochemistry 35 (1996) 2365–2373.
[7] I.M.-T.C. Crevel, A. Lockhart, R.A. Cross, J. Mol. Biol. 257 (1996) 66–76.
[8] Y.-Z. Ma, E.W. Taylor, Biochemistry 34 (1995) 13233–13241.
[9] S.T. Brady, Nature 317 (1985) 73–75.
[10] R.D. Vale, T.S. Reese, M.P. Sheetz, Cell 42 (1985) 39–50.
[11] S.S. Rosenfeld, B. Rener, J.J. Correia, M.S. Mayo, H.C. Cheung, J. Biol. Chem. 271 (1996) 9473–9482.
[12] T. Shimizu, E. Sablin, R.D. Vale, R. Fletterick, E. Pechatnikova, E.W. Taylor, Biochemistry 34 (1995) 13259–13266.
[13] S. Fujiwara, F.J. Kull, E.P. Sablin, D.B. Stone, R.A. Mendelson, Biophys. J. 69 (1995) 1563–1568.
[14] D.B. Murphy, G.G. Borisy, Proc. Natl. Acad. Sci. USA 72 (1975) 2696–2700.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[16] T. Shimizu, H. Morii, J. Biochem. 120 (1996) 1176–1181.
[17] D.D. Hackney, Proc. Natl. Acad. Sci. USA 85 (1988) 6314–6318.
[18] A. Lockhart, R.A. Cross, D.F.A. McKillop, FEBS Lett. 368 (1995) 531–535.
[19] S.P. Gilbert, M.R. Webb, M. Brune, K.A. Johnson, Nature 373 (1995) 671–676.
[20] J.W. Shriver, B.D. Sykes, Biochemistry 20 (1981) 2004–2012.
[21] T. Kodama, K. Fukui, K. Kometani, J. Biochem. 99 (1986) 1465–1472.
[22] T. Shimizu, K. Furusawa, Biochemistry 25 (1986) 5787–5792.