Interactions between Subunits in Heterodimeric Ncd Molecules*1

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The nonprocessive minus-end-directed kinesin-14 Ncd is involved in the organization of the microtubule (MT) network during mitosis. Only one of the two motor domains is involved in the interaction with the MT. The other head is tethered to the bound one. Here we prepared, purified, and characterized mutated Ncd molecules carrying point mutations in one of the heads, thus producing heterodimeric motors. The mutations tested included substitutions in Switch I and II: R552A, E585A, R579A, and E585D; the decoupling mutant N600K; and a deletion in the motor domain in one of the subunits resulting in a single-headed molecule (NcN). These proteins were isolated by two sequential affinity chromatography steps, followed by measurements of their affinities to MT, enzymatic properties, and the velocity of the microtubule gliding test in vitro. A striking observation is a low affinity of the single-headed NcN for MT both without nucleotides and in the presence of 5′-adenylyl-β,γ-imidodiphosphate, implying that the tethered head has a profound effect on the structure of the Ncd-MT complex. Mutated homodimers had no MT-activated ATPase and no motility, whereas NcN had motility comparable with that of the wild type Ncd. Although the heterodimers had one fully active and one inactive head, the ATPase and motility of Ncd heterodimers varied dramatically, clearly demonstrating that interactions between motor domains exist in Ncd. We also show that the bulk property of dimeric proteins that interact with the filament with a non-processive minus-end-directed motor Kar3 can associate with a non-motor polypeptide, Cik1 or Vik1, forming a dimer. Although neither Cik1 nor Vik1 can generate movement, they bind microtubules (5, 6). In kinesin NcK3, only one of the heads interacts with the microtubule and generates force (7). The x-ray derived structure of the NcK3 motor domain did not provide a clue as to the origin of the head asymmetry (8). Generally speaking, the reason for the occurrence of two heads in nonprocessive motors is unclear.

Ncd (non-claret disjunctional) is an often studied minus-end-directed motor in Drosophila melanogaster. The motor performs important functions during mitosis and meiosis (9, 10). Ncd has two identical heads, located at the C termini of the polypeptides, which are tightly attached to a superhelical segment. The N-terminal tail contains also a secondary ATP-independent MT-binding site (11, 12). Although many lines of experimental evidence support the view that Ncd is a nonprocessive motor, it was reported recently that full-length Ncd may exhibit some characteristics of a processive protein (13). However, this property was attributed to the interaction of the Ncd tail with a bundle of microtubules rather than a coordinated action of the motor domains. Our own investigation indicated that single molecules of Ncd do not move processively (14). Several studies using electron microscopy clearly indicated that only one of the Ncd motor domains binds to the microtubule (15–17). The other domain remains tethered to the bound one. Furthermore, it has been proposed that the Ncd mechanochemical cycle appears to consist of a single power stroke followed by a dissociation of the head from the MT (16). This is consistent with the finding that a single-headed Ncd, a dimeric protein containing one head, the neck, and part of the stalk, showed an almost unreduced motility (16).

Several experiments suggest that the heads of Ncd do not act independently. The rate-limiting step in the kinetics of the monomeric Ncd construct MC6 is the release of ADP, which in MC6 occurs with negligible amplitude compared with the dimeric construct MC1, suggesting that the partner head may...
help to accelerate the dissociation of the motor domain from the microtubule (18). This result may be difficult to interpret because MC6 is lacking not only the head but also the neck, which may be crucial for the head release process. However, for the dimeric MC1 construct, a direct microtubule binding study demonstrated that interaction of nucleotides and phosphate with the phosphate site in one head increases the affinity of the site in the other, stimulating motor release from the microtubule (19). Consistent with these data were also the finding that the two identical heads of Ncd contain two sites for ADP that have different affinities (20), which can also be explained by proposing a strong negative cooperativity between heads.

In this paper, we have prepared, purified, and characterized heterodimers of Ncd carrying a point mutation in one of the heads and no mutation in the other. The mutated amino acids were key residues that are involved in the mediation of structural changes in the head that follow the hydrolysis of the nucleotide (Fig. 1). It is generally accepted that kinesin motor domains sense the presence of the ATP γ-phosphate and switch between ATP, ADP-P, and ADP conformations, which, in turn, have different microtubule affinities and different structures of the force-generating components. Two clusters of highly conserved residues are involved in these processes: Switch I (residues NXXSSR) in Ncd residues 547–552, and Switch II (DLAGSE) comprising Ncd amino acids 580–585 (21, 22). We have mutated Arg 552 to Ala or Glu 580 to Ala or Asp. We have also replaced Asn 600 with Lys. Asn 600 is essential for integrity of the Ncd dimeric structure and transmission of conformational changes from the switch region to the microtubule-binding site. It is located in a hydrogen-bonded turn adjacent to the N terminus of the microtubule-interacting helix α4 (Fig. 1). In dimeric Ncd, the mutation results in a rather unusual, asymmetrical conformation, where one of the motor domains is detached from the neck and rotated with the superhelical segment by about 75° (23). It has been postulated that such conformation of the motor molecule exists during the mechanochemical cycle of Ncd (16, 23). All of these mutants in the monomeric form of Ncd retain their basal, very low ATPase activity but show a negligible microtubule-stimulated ATPase (23, 24). Therefore, we expect that, like Ncd-N600K (23), homodimeric forms of Ncd-E585A or -E585D or Ncd-R552A would be inactive both in vitro motility tests and as microtubule-activated ATP hydrolyzing enzymes. However, the effect of one functionally inactive head on these properties depends on possible interactions between motor domains of dimeric Ncd.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—We developed a two-plasmid expression system for Ncd; one of the plasmids (a derivative of pET28a (Novagen)) directed expression of a subunit with an N-terminal His tag (NH2-MGSSHHHHHHHSSGLVPRG-SHM-); the other (a derivative of pBIOEx) directed expression of a subunit with a short amino acid sequence (NH2-MAGGLNDIFEAQKIEWHE) that in *Escherichia coli* is biotinylated in vivo at the underlined lysine (25). An Ndel-XhoI fragment from plasmid pNCDET (26) containing sequence coding for Ncd fragment 250–700 was recloned into pET28a (+) vector (Novagen), resulting in plasmid pNCDET28 expressing the Ncd sequence with an N-terminal fusion of His tag. To obtain biotinylated Ncd fragment 250–700, the appropriate part of the Ncd coding sequence was amplified with primers BIONcDN (5′-TACTCGAGCGACAACGAGTGTCTTCAGAGG-3′ and NcDR (5′-AGCTCGAGTTATTTATCGAACTCGGAGGTGTCTTCAGAGG-3′) and NcDR (5′-AGCTCGAGTTATTTATCGAACTCGGAGGTGTCTTCAGAGG-3′) XhoI recognition sequences underlined), cut with XhoI and ligated into pBIOEx, digested with XhoI, and dephosphorylated with calf intestinal alkaline phosphatase. Recombinant plasmid with the insert ligated in a proper orientation, pBIONcD, was selected based on restriction analysis. Plasmid pBIONcDN expressing a biotinylated fragment of Ncd neck (aa 250–347) was constructed by PCR-generated deletion in pBIONcD with primers NcdBioNf (5′-TACCTCGAGCTCAAGTCCATGAAGG-3′) and NcdBioNr (5′-TACCTCGAGCTCAAGTCCATGAAGG-3′). PCR product was phosphorylated with T4 polynucleotide kinase, blunt end-ligated with T4 DNA ligase, and transformed into bacterial cells. Plasmid pHisNdN expressing the same fragment of Ncd with the N-terminal His tag was constructed in a similar way from pNCDET28 with primers NcNf (5′-TGCACACGGCCCAAGATGAAAC-3′) and NcNr (5′-AGCTCGAGTTAGCCCGGAGGTCCATGAAGG-3′). Mutations were introduced into pNCDET28 construct by PCR using *Pfu* polymerase (Fermentas). PCR products were full-length recombinant plasmids with point mutations. In some cases, another silent mutation changing the restriction map of mutagenesis product was introduced to facilitate initial screening (primers used in site-directed mutagenesis are shown in supplemental Table S1). PCR products were then treated in the same way as described above for construction of pBIONcDN. All new plasmid constructs used in this work were confirmed by sequencing.

**Protein Expression and Purification**—All constructs were expressed in *E. coli* BL21(DE3) pLys strain (Novagen) freshly transformed with the plasmids. Heterodimers were obtained by
cotransformation with two plasmids, one expressing the His-tagged subunit (pET28a derivative) and another expressing the biotinylated subunit (pBIoEx derivative). Heterodimeric kinases are described throughout this work with the His-tagged subunit first and the biotinylated subunit second (i.e. N600K/WT is a heterodimer containing a His-tagged mutant subunit and a WT biotinylated subunit). All constructs contained aa 250–700 of full-length Ncd protein except NcN and NcN-E585D, which contained aa 250–700 in one subunit and 250–347 in the other.

Bacteria were grown in Luria broth medium containing kanamycin (50 μg/ml) and, where appropriate, also tetracycline (10 μg/ml) and biotin (50 μg/ml). Saturated overnight cultures were diluted 1:20 with fresh medium and grown to mid-log phase. The temperature was then decreased to 25 °C, and isopropl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. Cells were harvested after 3–5 h of induction in the case of homodimeric proteins and after 14 h in the case of heterodimeric constructs. Bacterial pellets were washed with buffer A (20 mM HEPES, pH 6.9, 1 mM MgCl2, 10 mM 2-mercaptoethanol, 300 mM NaCl, pH 7.2) plus 20 mM imidazole and resuspended in the same buffer supplemented with a protease inhibitor mixture (1 mM phenylmethanesulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 2 μg/ml aprotinin) and DNase/RNase, disrupted by a single passage through a French press (Thermo Spectronic) at 1380 bars, and then centrifuged (18,000 rpm, 30 min). Clarified lysates were loaded onto appropriate columns.

A two-step affinity purification was performed to separate heterodimeric constructs from other species. First, the lysate in buffer A plus 20 mM imidazole was loaded on a column with Ni2+-nitriloacetic acid-agarose resin (Sigma). The column was washed with buffer A plus 20 mM imidazole, and His-tagged protein was eluted with buffer A plus 300 mM imidazole. The fractions containing His-tagged proteins (homodimers and heterodimers) were loaded onto a monomeric avidin-Sepharose column (Affiland, Liège, Belgium) to separate heterodimeric protein from His-tagged homodimers, and after washing with buffer B (10 mM HEPES, 1 mM MgCl2, 1 mM EGTA, 10 mM, 2-mercaptoethanol, pH 7.2) plus 200 mM NaCl and 1 mM ATP, the heterodimers were eluted with buffer B plus 100 mM NaCl, 5 mM biotin, and 1 mM ATP.

The His-tagged homodimeric Ncd was isolated in a single affinity purification step on an Ni2+-nitriloacetic acid-agarose column. The elution was done with buffer A, 100 mM NaCl, 300 mM imidazole. Only peak fractions were collected and frozen at −70 °C in the presence of sucrose (10% (w/v)). Homogeneity of all preparations was confirmed on SDS-PAGE with Coomassie stain (Fig. 2A). Ncd concentration was determined spectrophotometrically using an ε280 of 27,960 M−1 cm−1 (calculated from the amino acid composition). In all experiments reported here, the protein concentration was expressed “per Ncd head.”

Western blot analysis was performed to further confirm the identity of purified proteins (Fig. 2, B and C). Samples were separated in 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature using a solution containing 5% nonfat milk, 0.2% Tween 20, and 0.05% sodium azide in 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4. His-tagged proteins were visualized after a 1-h incubation with a 1:500 dilution of anti-His tag antibody (Qiagen). The primary antibody was detected using a 1:10,000 dilution of anti-mouse antibody conjugated with alkaline phosphatase (Sigma). Visualization of biotinylated proteins was done using streptavidin conjugated with alkaline phosphatase (Sigma), diluted 1:35,000 in Tris-buffered saline plus 0.2% Tween 20.

**Purification and Polymerization of Tubulin**—Porcine brain tubulin was purified and polymerized as described previously (26). Microtubule concentration is given as the concentration of α,β-tubulin heterodimer estimated by the Bradford method, using bovine serum albumin as a standard.

**Measurement of MT-induced Release of mantADP from Ncd Constructs**—All experiments were performed in buffer N (20 mM HEPES/KOH, 100 mM potassium acetate, 5 mM magnesium acetate, 0.1 mM EGTA, 0.1 mM EDTA, 5% sucrose, pH 7.2) and 20 μM paclitaxel at 20 °C. Ncd constructs were incubated with a 4-fold excess of mantATP (Molecular Probes) for 1 h at 4 °C. Excess of the nucleotide was removed using a NAP-10 column (GE Healthcare), and the spectra were measured in a Hewlett-Pack-
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and 8452A spectrophotometer. The fluorescent motor-mantADP complex (0.4 μM) was placed in a 0.4 x 1-cm quartz cuvette (Hellma), and its emission intensity was measured in a SPEX Fluorolog-3 fluorometer, using the excitation and emission wavelengths of 340 and 440 nm, respectively. Then MTs (5 μM) were added and rapidly mixed, and the release of mantADP was monitored. At the end, nucleotides (ATP, AMPNNP, or ADP) were added, and the second phase of the mantADP release was observed. All experiments were performed at least in triplicate for each protein. For fluorescence polarization, fluorescent motor-mantADP complexes (1 μM) were placed in a small quartz cuvette (0.4 x 1 cm), and the polarization of fluorescence was measured using automatic polarizers of SPEX Fluorolog-3 every 3 min for 30 min for each construct. The excitation and emission wavelength were 340 and 440 nm, respectively.

Multiple Motor Motility Assay—Microtubules were polymerized from 5 μl of porcine brain tubulin labeled with tetramethylrhodamine in buffer BRBB80 (80 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA) with 4 mM MgCl2, 1 mM MgGTP, and 5% dimethyl sulfoxide at 37 °C. After 30 min, the microtubule polymers were stabilized and diluted 100-fold at room temperature in BRBB80 buffer containing 40 μM paclitaxel. A penta-His antibody (Qiagen) was fixed on the silanized surface of a coverslip (27). The chamber was washed with BRBB80 buffer to remove unbound antibody and then filled with 1% F127 blocking polymer (Sigma). Before adding Ncd, the chamber was filled with buffer N containing 1 mM ATP. Next, Ncd homodimers or heterodimers in buffer N were added into the flow chamber. After removing unbound Ncd, tetramethylrhodamine-labeled MTs were introduced in buffer N and supplemented with anti-fades (20 mM d-glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase, 1 mM dithiothreitol), 1 mM ATP, and 10 μM paclitaxel. MTs were observed using a fluorescent microscope Jenalumar (Jena, Germany) equipped with a camera (C2400, Hamamatsu). The motility assay was carried out at 25 °C. Filament velocity was measured on digitized video sequences (usually 150 frames), collected with a frame grabber (PixelSmart 512PCI). Typical lapse time between the digitized frames was 2 s. The mean velocity was calculated for all filaments in the field of view using the Retrac program (shareware by Nick Carter; available on the World Wide Web).

Ncd Affinity to Microtubule—All experiments were performed in buffer N with appropriate nucleotide (0.5 mM ADP or 0.5 mM AMPNNP) and 10 μM dithiothreitol. Ncd homodimers were labeled with a 4-fold molar excess of 1,5-IAEDANS for 1 h at 4 °C. Excess of the dye was removed on NAP-10 column, and the absorption spectrum was measured. The labeling stoichiometry was 0.93 ± 0.16. 1,5-IAEDANS-labeled Ncd at 0.4 μM was mixed with MTs from 0 to 10 μM. The mixture was incubated for 15 min at room temperature and then centrifuged (80,000 rpm, 30 min, 20 °C) to sediment the Ncd-MT complex. The supernatant was collected, and the amount of unbound Ncd was estimated by measurement of fluorescence intensity (excitation, 340 nm; emission, 500 nm). The values of Kd, the dissociation constant for the Ncd-MT complex, were determined by fitting the cosedimentation data to the equation,

\[ f_b = \left( (P_0 + K_d + M_{T0}) - (P_0 + K_d + M_{T0})^2 - 4P_0M_{T0}(1/2) \right)/(2P_0) \]  

where \( f_b \) represents the fraction of MT-bound protein (Ncd). \( f_b = (F_0 - F_c)/F_c \), where \( F_0 \) and \( F_c \) denote the values of fluorescence intensities of 1,5-IAEDANS-Ncd in the supernatant at the total tubulin concentration equal to 0 and \( M_{T0} \), respectively. \( P_0 \) and \( M_{T0} \) are the total Ncd and microtubule concentrations, respectively.

ATPase Assay—The rates of ATP hydrolysis by Ncd were determined spectrophotometrically by monitoring the oxidation of NADH enzymatically coupled to the turnover of ATP by lactate dehydrogenase, pyruvate kinase, and phosphoenolpyruvate, as described before (28). Assays were performed in buffer N, supplemented with 0.1 mM dithiothreitol and 20 μM paclitaxel at 25 °C. ATP was used at a final concentration of 2 mM, and the reactions were started by the addition of a constant amount of motor protein (usually 0.2 μM) and different concentrations of microtubules (0–10 μM). Individual rates were plotted against increasing MT concentrations, and the values of \( K_{d3}(MT) \) and \( k_{cat} \) were obtained by least squares fitting of the data to rectangular hyperbolas using either the SigmaPlot application (Jandel) or a custom-written program, which employed the Marquardt-Levenberg algorithm.

Binding of Heterodimeric Ncd to MT—With two nonequivalent heads, binding of dimeric Ncd molecule to the microtubule results in the formation of two complexes PM1 (binding through Head 1) and PM2 (binding through Head 2) with the respective dissociation constants \( K_{d1} \) and \( K_{d2} \). The constituents of these complexes exist in rapid equilibrium. We made three assumptions as follows. 1) At any time, the Ncd molecule can bind only one microtubule. We consider that a ternary complex consisting of one Ncd molecule bound simultaneously to two MTs would not be sterically feasible. 2) Ncd interacts with the microtubule using only one of its heads. 3) The interactions between the mutated and WT head are the same as those between two WT heads, at least as far as the interactions between the motor and MT are concerned. In the equations below, \( P_0 \) and \( P \) denote total and free concentration of the protein (Ncd), and \( M_{T0} \) and \( MT \) refer to the total and free microtubule concentration. \( P + MT \rightleftharpoons PM_1; K_{d1} = P\cdot MT/PM_1; P + MT \rightleftharpoons PM_2; K_{d2} = P\cdot MT/PM_2; M_{T0} = MT + PM_1 + PM_2 = MT + P\cdot MT/(1/K_{d1} + 1/K_{d2}). \)

Substituting \( \theta = 1/K_{d1} + 1/K_{d2} \), one obtains the quadratic equation, \( \theta P^2 + (1 + \theta M_{T0} - \theta P_0) P - \theta P_0 = 0 \), substituting \( \lambda = 1 + \theta M_{T0} - \theta P_0 \), if the discriminant \( (\lambda^2 + 4\theta P_0) > 0 \), then one obtains the solution, \( P = (-\lambda + (\lambda^2 + 4\theta P_0)^{1/2})/(2\theta) \) and the following.

\[ PM_1 = P\cdot MT_0/(K_{d1}(1 + \theta P_0)) \]  

\[ PM_2 = P\cdot MT_0/(K_{d2}(1 + \theta P_0)) \]  

It is also useful to note the following relation.

\[ PM_1/PM_2 = K_{d2}/K_{d1} \]
We also note that the presence of homodimers in the preparations of heterodimers would affect only the kinetic measurements because the Ncd molecules that lacked at least one His tag would be washed off during preparation of the chamber in which the Ncd is immobilized by anti-His tag antibody. To examine the possibility that the heterodimers exchange their subunits, thus reforming homodimers, we monitored fluorescence resonance energy transfer between dyes attached to Cys\(^670\) residues located on the motor domains of the homodimer mixtures (see supplemental material). The conclusion of these experiments was that the dissociation constants are summarized in Table 1. The mutations had a divergent effect on the Ncd affinity. For most of the mutants, the affinity in the presence of nucleotides, is considerably lower. One exception is the Ncd-R552A and Ncd-N600K, which displayed relatively low microtubule affinity in the AMPPNP state, and have been shown to be unaffected by our biochemical assays. Pseudoheterodimer (dimmer Ncd with two WT heads and different tags) had properties indistinguishable from those of the WT Ncd (results not shown).

We determined the affinity of WT and mutated Ncd homodimers for MT in the absence and in the presence of ADP and AMPPNP using the cosedimentation assay (Table 1). Rather than employing gel densitometry, we have labeled Ncd with 1,5-IAEDANS on Cys-670 and measured fluorescence of the dye in the supernatant after a 30-min centrifugation. Modification of Cys\(^670\) in Ncd with thiol-specific labels is specific, resulting in \(\sim 1\) molecule of the dye incorporated per subunit (29, 30). The modification does not change significantly the kinetic parameters of the protein ATPase for monomeric (29) and dimeric (26) constructs. To further confirm that the modified Ncd retained its basic characteristics, we have also measured the motility of the modified protein in the multiple motor gliding assay. The velocity of Cys-labeled Ncd was 84% of that measured for the unmodified protein. There are many advantages of this assay over classical cosedimentation and analysis of the pellet and supernatant by PAGE densitometry. The motor can be used in a much wider range of concentrations, and the data are not influenced by the presence of unpolymerized tubulin (the subunit molecular mass of our Ncd construct is about 50 kDa, almost the same as each of the tubulin subunits).

The values of the dissociation constants are summarized in Table 1. The mutations had a divergent effect on the Ncd affinity. For most of the mutants, the affinity for the MT, especially in the presence of nucleotides, is considerably lower. One exception is the E585A, which had higher affinity for the MT, especially in the presence of ADP (Table 1). Another interesting observation was an order of magnitude lower affinity of NcN in the absence of nucleotides compared with the dimeric Ncd form (Fig. 3). An unexpected result for this single-headed motor, however, was its very low affinity for the MT in the presence of AMPPNP. Although several other mutants, such as the R552A and Ncd-R600K, also displayed relatively low microtubule affinity in the AMPPNP state, we have to remember that in contrast to those mutants, NcN displays good gliding velocity in vitro. Since only one of the Ncd heads is involved in binding to the microtubule, the effect of the second head in the dimeric motor must involve either residual binding of the tethered head to the microtubule, or, what is more likely, the formation of the bound domain is altered by the presence of the second head.

Steady-state ATPase of Ncd Heterodimers—With the exception of the E585D and NcN-E585D, all other homodimeric
TABLE 2

Steady-state ATPase activities and gliding velocities of Ncd heterodimers

| Ncd construct       | $K_{d,1}(\text{MT})$  | $k_{cat}/\text{head}$  | $v$ |
|---------------------|------------------------|------------------------|-----|
| WT/WT               | 0.8 ± 0.2              | 3 ± 0.07               | 140 ± 20 |
| E85A/WT             | 1.9 ± 0.8              | 0.2 ± 0.03             | 1   |
| E85A plus WT mix    | 0.6 ± 0.1              | 0.7 ± 0.01             | 15 ± 4 |
| R552A/WT            | 1.9 ± 0.1              | 0.6 ± 0.02             | 65 ± 14 |
| R552A plus WT mix   | 2.2 ± 0.1              | 0.6 ± 0.01             | 30 ± 12 |
| N600K/WT            | 4.7 ± 0.8              | 1.4 ± 0.10             | 95 ± 24 |
| N600K plus WT mix   | 0.4 ± 0.1              | 0.6 ± 0.03             | 100 ± 17 |
| E85D/E85D           | 1.1 ± 0.4              | 0.19 ± 0.01            | 5 ± 0.2 |
| E85D/WT             | 5.2 ± 1.8              | 0.9 ± 0.2              | 20 ± 3 |
| E85D plus WT mix    | 1.0 ± 0.1              | 0.7 ± 0.02             | 11 ± 2 |
| NcN                 | 13.7 ± 4.5             | 1.9 ± 0.5              | 125 ± 28 |
| NcN-E85D            | ND*                    | 0.1 ± 0.01             | ND* |
| NcN-E85D plus WT mix| ND*                    | 0.6 ± 0.1              | ND* |

*ND, not determined; $k_{cat}$ corresponds to the ATPase at 5 μM MT.

Mutants showed negligible microtubule-activated ATPase activity. The values of $k_{cat}$ and $K_{d,1}(\text{MT})$ for the heterodimers are shown in Fig. 4 and summarized in Table 2. Because of the reports that Ncd can cooperatively bind MT (17, 31), we have also determined the steady-state ATPase activity for 1:1 mixtures of the mutated and wild type motors. For an equimolar mixture of WT and inactive motors, one expects $k_{cat}$ to be exactly equal to 50% of the WT ATPase (i.e. 0.66 s⁻¹). Because the homodimer Ncd-E85D has a residual ATPase, a value of 0.76 s⁻¹ is expected for the mixture of WT and Ncd-E85D. Perusal of Table 2 indicates that, within experimental error, this condition is fulfilled.

Both the ATPase and the motility for the heterodimers depend on two factors: 1) which head is bound to the microtubule and 2) whether or not the heads communicate with each other. Regardless of the molecular model employed to interpret the data, we have to take into account the fact that there exist two populations of the Ncd heads (WT and mutated) exhibiting different affinities. The relative concentrations of the MT complexes with Head 1 and Head 2 are governed by the law of mass action and usually are not equal, because for heterodimers $K_{d,1}$ is different from $K_{d,2}$. Therefore, it is not justified to think that a heterodimer composed of one fully active and one inactive head will have 50% of the activity of the WT motor.

Furthermore, we have to distinguish between two types of the intersubunit interactions. The first occurs when the presence of Head 2 statically alters the conformation of Head 1 (e.g. by increasing the affinity of Head 1 for the MT). NcN may serve

![FIGURE 4. Steady-state ATP hydrolysis by Ncd homo- and heterodimers.](image)

Had no MT-stimulated ATPase activity. Note that the $k_{cat}$ values for the mixtures of homodimers were very similar, but those for the corresponding heterodimers were not. C, the fraction of heads bound by the WT head depends on the ratio of $K_{d,1}$ values for the heads. In the example, the $K_{d,1}$ (for WT head) was assumed to be 0.43 μM (see Table 1), and the value of the $K_{d,1}$ for the second head is given beside each curve. When the affinity of the second head is about an order of magnitude lower, the use of that head is almost completely eliminated.
Fluorolog polarizers every 3 min for 30 min for each construct. Presented in Fig. 4, we have simulated the ATPase of a heterodimers composed of one native and one mutated head, and the results are consistent with Fig. 4C. Next, we measured $K_{\text{cat}}$ (Fig. 4B) and observed striking correlation between the affinity of the mutated head in the heterodimer and a decrease or increase of the expected 50% reduction in the ATPase for the heterodimer. Starting with Ncd-E585A, we note that its free mantADP/head Anisotropy \( k \) (no MT)\(^a\) Fluorescence change upon binding to MT

| Ncd homodimer | mantADP/head | Anisotropy | \( k \) (no MT)\(^a\) | Fluorescence change upon binding to MT |
|--------------|--------------|------------|-----------------|-----------------------------------|
| WT | 0.63 ± 0.13 (n = 11) | 0.273 ± 0.010 (n = 8) | 0.0010 | NA |
| E585D | 0.70 ± 0.10 (n = 4) | 0.275 ± 0.004 (n = 4) | 0.0017 | NA |
| N600K | 0.67 ± 0.09 (n = 3) | 0.254 ± 0.008 (n = 7) | 0.0024\(^b\) | ND\(^c\) |
| R552A | 0.56 ± 0.16 (n = 3) | 0.262 ± 0.006 (n = 7) | 0.0011 | ND\(^c\) |
| E585A | 0.48 ± 0.15 (n = 2) | 0.04 (n = 4)\(^f\) | >0.05 | 0 |
| NgN | 0.98 ± 0.08 (n = 2) | 0.214 ± 0.005 (n = 2) | 0.0013 | ND |
| Free mantADP | NA\(^d\) | 0.028 ± 0.002 (n = 8) | NA | NA |

\(^a\) Rate constant for nucleotide dissociation in the absence of MT. S.E. for these measurements was 2.5 \times 10^{-4} s^{-1}.
\(^b\) Data from Ref. 23.
\(^c\) ND, not determined.
\(^d\) The initial value of anisotropy was 0.06 and decreased to 0.04 in less than 5 min.
\(^e\) NA, not applicable.

as an example. Several key properties of Ncd depend on the presence of the second head. The second (dynamic) type takes place when the effects of ligand interactions with Head 1 are transmitted to Head 2. This type of interaction probably exists for ADP binding to dimeric Ncd in the presence but also in the absence of MT (20). These two types are not mutually exclusive. However, the effects seen for the interaction of the dimeric Ncd with the MT are not of the dynamic type because when one of the heads forms a complex with the MT, the other is prevented from binding. Therefore, if Ncd binds with Head 1 attached to the microtubule, it is immaterial if the binding effect is transmitted to Head 2 because Head 2 will not bind to the MT anyway. The interactions between the heads should be taken into account if the two heads-bound intermediate occurs at a significant concentration. However, the existence of this intermediate in Ncd has never been demonstrated experimentally. Its occurrence would require a substantial unwinding of the Ncd coiled coil or a secondary binding site for the motor on the microtubule. Although we consider it unlikely that this species constitutes a dominant or even significant fraction of the bound motors, we note that the processive kinesin Eg5 may begin its run with both motor domains interacting with the microtubule (32).

Our general strategy was to separate the effects of the unequal affinity of the heads from the intramolecular interactions in the dimeric Ncd molecule. In the first approximation, we assumed that the interactions between the mutated and WT head are the same as between two WT heads. Using Equations 3 and 4, we have simulated the ATPase of a heterodimers composed of one native and one mutated head, and the results are presented in Fig. 4C. Next, we measured \( K_{\text{cat}} \) (MT) and \( k_{\text{cat}} \) (Fig. 4B) and observed striking correlation between the affinity of the mutated head in the heterodimer and a decrease or increase of the expected 50% reduction in the ATPase for the heterodimers. Starting with Ncd-E585A, we note that its \( K_p \) (AMPPNP) is about 0.2 \( \mu \text{M} \), implicating tighter binding and a population of bound motors biased toward species interacting through the inactive head. From Fig. 4C we may conclude that for the E585A/WT heterodimer, about 25–30% of the heads would have the WT head attached to the microtubule. The heterodimer E585A/WT exhibits 16% of the WT activity (Fig. 4A). For N600K heterodimer the \( K_p \) (AMPPNP) is 7.3 \( \mu \text{M} \); therefore, from Fig. 4C, we expect that the binding occurs almost exclusively by the WT head, so the effect of the inactive motor domain is negligible, and apparently the heterodimer behaves as if the WT head somehow overpowered the effect of the inactivating mutation in the other head. Since a set of equilibrium binding constants is insufficient to build a structural model of Ncd ATPase, one should not expect an excellent agreement between the experimental and simulated data. Nonetheless, the observed trend is quite clear indicating that the Ncd heads are enzymatically interactive, but this interaction is not dramatic.

**ADP Release from Ncd Homodimers by Microtubules and Nucleotides**—When Ncd is incubated with an excess of mantATP, the nucleotide resident at the active site is replaced by the fluorescent analog. Since the basal ATPase is not equal to zero, mantATP is hydrolyzed to mantADP and P_i (see supplemental material). Binding of WT Ncd-mantADP complex to the microtubule results in a rapid release of the nucleotide to the solution what produces approximately a decrease of 20–40% in the fluorescence intensity of the mant chromophore (33, 34). It is believed that the dissociation of the nucleotide occurs only from the head that interacts directly with the microtubule. The addition of a hydrolyzable nucleotide or ADP to this complex enables a replacement of the nucleotide also in the partner head with a concomitant drop in the fluorescence intensity. We have tested the ability of the mutated Ncd to undergo such changes.

After the exchange, the mantADP-Ncd complex was passed through a small gel filtration column to remove the excess of the nucleotide. The ratio of mantADP per head bound to Ncd and the fluorescence anisotropy \( r_{340} \) were determined immediately after this treatment and are summarized in Table 3. With the exception of Ncd-E585A, the fluorescence intensity of mantADP in these complexes decreased slowly in time (\( k \approx 0.001–0.002 \text{ s}^{-1} \); Table 3), showing that they retain their integrity also after eliminating the external nucleotide. The rate of mantADP release from the dimeric Ncd-N600K in the absence of microtubules was measured before (23) and found to be slightly accelerated compared with the WT Ncd (\( k \approx 0.0024 \text{ s}^{-1} \)). Here, we measured the rate of mantADP release for Ncd-R552A. Despite
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The low incorporation level of the fluorescent nucleotide (Table 3), its release rate in the presence of MT was found to be only slightly faster than for the WT Ncd ($k = 0.0011 \text{ s}^{-1}$). Thus, the nucleotide that remained in Ncd-R552A is not loosely bound to the protein. The amplitudes of the mant fluorescence decrease on binding to the microtubule was measured for the WT and E585D Ncd and was found to have rather similar values: 22.5 ± 4.2 and 25.2 ± 2.3%. After the addition of 1 mM ATP, the fluorescence dropped further by 21 ± 3.3 and 18.2 ± 4.1% for WT and E585D, respectively. In conclusion, Ncd-E585D exhibited a mantADP release pattern very similar to that of the WT Ncd.

For Ncd-E585A, the value of the fluorescence anisotropy, $r_{340}$, immediately after removing the excess of mantATP was 0.06 and decreased to 0.04, indicating that in this case, most of the nucleotide was released rapidly from the protein. Since the dissociation of the nucleotide from the active site of Ncd leads to a rapid denaturation of the protein, the low anisotropy value is consistent with a high propensity of Ncd-E585A to aggregation (data not shown). In agreement with this finding, the fluorescence of the mantADP-Ncd-E585A complex was insensitive to the presence of MT (Table 3).

Multiple Motor Gliding Assay of Ncd Heterodimers and Mixtures of Homodimers—Motors tightly attached to the microtubule work and interact with the filament asynchronously. However, there exists mechanical coupling between them because many motors are attached to the same MT. This coupling occurs not only for heterodimers but also for mixtures of homodimers. To investigate possible interactions between motor domains (heads) of Ncd, we had to dissect the effect of the mutation on the behavior of the protein from the intersubunit interactions. We considered that a 1:1 mixture of WT and mutated protein would take into account the effect of the mutated protein per se on the motion of the microtubule. The velocities of the Ncd heterodimers and mixtures of WT and mutated homodimers were measured in the motility gliding assay in vitro. To exclude the possibility that differences in motility among Ncd mutants were caused by a variable number of motors interacting with the microtubule, we first measured the gliding velocities of the heterodimers in the range of concentrations exceeding 2 orders of magnitude. Fig. 5A shows that the velocities were constant, indicating the absence of cooperative behavior between motors that was seen for NcKin3 (7).

For each of the mutant proteins, we measured the gliding velocity of moving microtubule by the homodimer and found all of them but Ncd-E585D and NcN-E585D to be immotile. The measurements of the motility in the multiple motor gliding assays for the heterodimers and mixtures of homodimers are summarized in Fig. 5B. Clearly, the heterodimers behaved in a...
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Nonetheless, the latter possibility cannot be entirely ruled out because an additional Ncd site was reported on zinc-induced tubulin sheets (38).

The affinity of NcN for the microtubule in the presence of AMPPNP is so low that it is possible that after reorienting the neck/stalk segment, the motor dissociates from the filament. The question remains open whether the departure of the orthophosphate from the active site is necessary to trigger the dissociation of the Ncd motor domain from the MT. It is conceivable that for some of the mutants characterized here, the nucleotide is hydrolyzed after dissociation of the motor from the microtubule. Such a succession of events was postulated for kinesin-13 MCAK (39). It has been reported recently that the mitotic kinesin Eg5 as a single-headed motor is almost as effective in generating force as its double-headed form (40). Although Eg5 is processive, it has been argued that for kinesins that work in an ensemble of mechanically coupled motors, such as Eg5 or Ncd, the power stroke is a more effective mechanism than the processive walking in the production of movement (40).

Enzymatic and Motor Activities of Ncd Heterodimers—Here for the first time we isolated and characterized Ncd heterodimers with inactivating mutation in one of the heads. Heterodimers are employed more and more often to explore the role of the quaternary structure of molecular motors (25, 40–43). In most cases, the results that concern the bulk property of the heterodimers, such as the enzymatic activity or velocity in the gliding assay, are rather difficult to interpret. We pointed out clearly that the measured overall parameter for dimeric proteins that interact with the filament with only one of their heads, depends not only on the property of the constituent domains but also on the distribution of the filament-interacting subunits. In our experiments, this has been taken into account by measuring the motility of the equimolar mixture of two homodimers with inactivating mutation in one of the heads. Although Eg5 is processive, it has been argued that for kinesins that work in an ensemble of mechanically coupled motors, such as Eg5 or Ncd, the power stroke is a more effective mechanism than the processive walking in the production of movement (40).

To investigate further this point, we created NcN-E585D, a single-headed Ncd construct carrying the mutation E585D on its head, and examined it in the ATPase and multiple motor gliding assays. Since the construct was found to be fragile and susceptible to aggregation, we did not characterize it thoroughly. We only determined that the motor had low microtubule-stimulated ATPase (Table 2) and, when tested alone, no motility. When the ATPase of the equimolar mixture of NcN-E585D mutant and WT Ncd was measured, a rather expected value of 0.61 ± 0.01 s⁻¹ (Table 2) was obtained. In the gliding assay of the mixture of the WT and single-headed mutant NcN-E585D, the motility was significantly inhibited (Fig. 5B), albeit to a lesser degree than that for the mixture of the WT and the dimeric mutant.

The motility generated by mixtures of motors was theoretically studied by Tawada and Sekimoto (35). Since the duration of the actual mechanical step is much shorter than the pausing time between the steps, most motors remain attached to the filament (microtubule or F-actin), exerting impeding force on the filament motion (“protein friction”). Tawada and Sekimoto (35) derived an equation that links protein friction to the velocity of the filament for an ensemble of motors. This kind of analysis was performed for all homodimers (Fig. 5C). Plots with parameter γ < 1 are concave downwards and are obtained for proteins exerting higher protein friction than the WT, such as mixtures of the homodimers (Ncd-R552A:WT and E585D:WT in Fig. 5C). Proteins with γ > 1 interfere less with the work of native heads and are represented by Ncd-N600K:WT in Fig. 5C.

**DISCUSSION**

Single- and Double-headed Modes of Generating Force—A noteworthy observation reported here is a low affinity of the single-headed NcN for the microtubule both without nucleotides and in the presence of AMPPNP. Because in this protein only one head interacts with the filament, this must imply that 1) in dimeric Ncd, the tethered head also has some contact with the MT, or 2) the conformation of the interacting head is altered by the presence of the second head. Although there is no direct proof, cryoelectron microscopy reconstructions favor structural change in the dimer rather than a new interacting site on the microtubule for the tethered motor domain (15, 36, 37).

Because several kinesins exist as dimers and use their heads in profoundly different manners, we tried to find indications in their structures and properties that could shed more light on the role of the second passive head. NcKin3 is a dimeric plus-end-directed motor. The amino acid sequences of the heads are identical, but only one of the motor domains participates in hydrolyzing ATP and MT binding (7, 44). The x-ray structure of the NcKin3 motor domain did not reveal any unusual features that could be linked to this unusual behavior of the motor domains (8). Comparison of the kinetic properties of mono-

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meric and dimeric NcKin3 constructs have led Adio and Woehlke (44) to propose that such an asymmetric domain structure is important for triggering the detachment of the dimeric motor after one kinetic cycle. This conclusion is quite similar to the explanation for kinetic differences between monomeric and dimeric Ncd constructs (18, 19).

Even more puzzling is the yeast kinesin Kar3, a member of kinesin-14 subfamily. This kinesin exists in several variants, usually forming a dimer with a non-motor polypeptide (Vik1 or Cik1). The partner (Vik1) of the motor subunit binds microtubules but lacks an ATP binding site (6). In this case, the motor domain must not only generate force and movement but also detach the second subunit from the MT. It is, therefore, surprising that the Kar3-Vik1 dimer moves efficiently and with a higher velocity than the Kar3-Kar3 homodimer (5, 6). It has been proposed that the role of Vik1 is indirect (i.e. its presence in the dimer enables recruitment of many copies of the Kar3-Vik1 complex to a particular region of the spindle) (6).

Switch I and II Salt Bridge—It is believed that Switches I and II control the release of the nucleotide from the kinesin active site. Two mutations, R552A and E585A were designed to prevent the formation of a salt bridge between Switch I and II. Such a salt bridge exists in many but not all members of the kinesin superfamily (24). In retrograde kinesin Kar3, which can be considered an Ncd analogue in yeast, the salt bridge is present, and the x-ray structures of the protein after substitutions of the corresponding residues to alanines were solved (24). Generally speaking, the overall structure of the mutant motor domain was preserved. However, relatively small but significant changes in loop L9, helices a3 and a4, were detected. In Ncd, x-ray crystallography indicated that a salt bridge between residues Arg552 and GluE585 is absent in the monomeric (45) and dimeric (46) Ncd forms. The present study revealed that the affinity of mantADP is significantly reduced for most mutants studied here and particularly for Ncd-E585A. Therefore, some kind of ionic/hydrogen bonding interaction between Switch I and II seems dispensable for the correct functioning of the motor. Such interaction is possible for Ncd-E585D but not for Ncd-E585A, and consequently Ncd-E585D is the only mutant that exhibits residual ATPase and motility (Figs. 4 and 5 and Table 2).

One of the most intriguing findings described here is a high protein friction for Ncd-E585D (Fig. 5C). Although the release of mantADP upon binding of the motor to the MT for this mutant was not distinguishable from the behavior of the native protein and in the equimolar mixture of Ncd-E585D and WT homodimers the ATPase was almost 50% of that for the WT, in the multiple motor MT gliding test, Ncd-E585D molecules formed a large impediment for MT movement. It is possible that for Ncd-E585D, a bond bridging Switch I and II was initially formed but with Asp instead of Glu in position 585. Therefore, the release of this bond was impossible or quite difficult, resulting in a motor molecule stuck on the microtubule, dissociating only infrequently and producing the observed protein friction. Interestingly, the effect of the E585D mutation on the Ncd motility was not contingent on the presence of the second head in the molecule.

In conclusion, by analyzing the velocity of Ncd-powered microtubule movement, we obtained several pieces of data that provide new insight into the role of the dimeric structure in Ncd. The Ncd heads appear to be enzymatically and mechanically interactive. This finding is corroborated by the earlier evidence based on kinetic measurements (18, 19). However, the available Ncd crystal structures (21, 23, 46) do not provide a clue as to a pathway of communication between the motor domains. Furthermore, the biological significance of the subunit interactions in Ncd is not yet clear. In cells, an ensemble of Ncd molecules attached to one microtubule by its ATP-independent binding site moves another microtubule (47). Under this condition, the double headed Ncd motors distinguish parallel and anti-parallel orientation of the microtubules they transport (14). The mechanism underlying this phenomenon is not, in principle, dependent on the presence of dimeric species. However, it remains to be experimentally confirmed whether the double- and single-headed Ncd molecules work in similar fashion when supporting the microtubule-microtubule sliding.

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