ELSEVIER

Biophysical Chemistry 92 (2001) 127–139

Biophysical Chemistry

www.elsevier.com/locate/bpc

NCD activation of tubulin polymerization

Stefan Highsmith[a,*], Michael Thoene[a], Elena Sablin[b], Katherine Polosukhina[a]

[a] *Department of Biochemistry, University of the Pacific, San Francisco, CA 94115, USA*
[b] *Department of Biophysics and Biochemistry, University of California, San Francisco, CA 94143, USA*

Received 2 April 2001; received in revised form 21 June 2001; accepted 29 June 2001

Abstract

Tubulin dimer (tT) was purified from turkey erythrocytes. The motor domain of *Drosophila* non-claret disjunctional protein, NCD(335–700), was expressed in *E. coli* and purified. At 37°C in the presence of GTP, the rate of polymerization of tT to microtubule (tMt) is accelerated over threefold by the presence of NCD(335–700). At 10°C, the rate of tT polymerization is increased from zero, within experimental error, in the absence of NCD(335–700) to rates near those observed at 37°C when NCD(335–700) is present. The NCD(335–700) concentration dependence of the rate indicated the reactive species was NCD(335–700)$_n$.tT, with $n \approx 2$. At 10°C in the absence of GTP, polymerization does not occur, but tT activates NCD(335–700) MgATPase activity 10-fold. For the same conditions, using mians-NCD(335–700), which is modified with 2-(4′-maleimidylanilino) naphthalene-6-sulfonic acid, the apparent K_D for binding to tT is 2.3×10^{-5} M in the presence of MgADP. Replacing ADP with AMPPNP or ATP has a negligible effect on K_D. Mians-NCD(335–700) binding to tMt is 10-fold stronger than to tT. The above data indicate NCD(335–700) binds at a functional site on tT. The stoichiometry is consistent with the formation of NCD(335–700)$_2$.tT which in vitro accelerates self-assembly initiation and/or polymerization by binding a second tT in a position favorable for tubulin–tubulin interaction. The data suggest that in vivo functional NCD binding to microtubule involves one motor domain binding to α- and β-subunits at the interface of two different tubulin dimers in a protofilament.

Keywords: Turkey tubulin; NCD; Binding; Fluorescent modification; Microtubule

Abbreviations: NCD(335–700), non-claret disjunctional protein motor domain, residues 335–700; tT, turkey erythrocyte tubulin α,β-dimer; bT, bovine brain tubulin α,β-dimer; tMt, turkey erythrocyte microtubule; bMt, bovine brain microtubule; β-ME, beta-mecaptoethanol; mians, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; MOPS, 3-[*N*-morphalino]propane sulfonic acid.

* Corresponding author. Tel.: +1-415-929-6670; fax: +1-415-929-6654.
E-mail address: shighsmith@sf.uop.edu (S. Highsmith).

0301-4622/01/$ - see front matter
PII: S0301-4622(01)00197-1

1. Introduction

Non-claret disjunctional protein (NCD) is a member of the kinesin family and is involved in mitotic and meiotic spindle morphogenesis and assembly [1,2]. It has an ATP-dependent microtubule binding site on each of its two motor domains, and an ATP-independent microtubule binding site on the distal globular N-terminus domain, which is separated from the motor domains by an α-helical coiled-coil stalk and neck region [3]. In vitro, NCD is a minus-end directed microtubule-based motor [4,5], which is weakly if at all processive [6–8]. In vivo, it is required for normal meiotic spindle development and function, although its exact role is not known. It may be required for microtubule bundling, or sliding, or spindle morphogenesis [1,2,9].

There are other members of the kinesin family of microtubule-based motors that have motor domain structures similar to NCD, but different properties [10]. Conventional kinesin is a highly processive dimeric motor that moves toward the plus end of a microtubule [11]. Its role is to move vesicular cargo. XKCM1, also dimeric, is a non-motile species that binds at the ends of microtubules and uses ATP hydrolysis energy to promote depolymerization [12,13]. Kar3 is a minus-end directed, non-processive, monomeric motor [14], which forms heterodimers with regulatory proteins that determine whether it stabilizes or destabilizes microtubules [15]. NCD stabilizes microtubules [16]. In spite of their differing functions, the motor domains of kinesin, NCD and Kar3 have similar high-resolution structures [17–19]. A single-headed member of the kinesin family, KIF1A, is surprisingly processive, by virtue of an extended electrically charged loop on the motor domain that can bind to the oppositely charged C-terminal flexible chain on the α-subunit of a neighboring tubulin dimer in the microtubule protofilament [20,21].

These diverse interactions of microtubules with structurally similar kinesin family members highlight that microtubules are dynamic components of the cytoskeleton, in addition to providing tracks on which some of the motors move. Microtubules grow most rapidly at the plus end, where a cap of tubulins that have GTP bound at the E-site of the β-subunit protects the assembly from episodes of catastrophic depolymerization [22–24]. The roles of some of the motors of the kinesin family are to regulate microtubule position, growth and function. NCD may be among these.

The atomic structure of a monomeric NCD motor domain construct NCD(335–700) has been solved [18], and there are electron micrograph image reconstruction structures of NCD(335–700) bound to microtubule [25–27]. The affinity of NCD for microtubule is high [28]. The kinetics of the basal and microtubule-activated MgATPase cycle in solution has been investigated for the monomeric motor domain [29–31]. The results indicate that the hydrolytic cycle includes microtubule-bound and unbound states, as expected for a motile motor protein system.

A high-resolution structure for microtubule was solved by electron diffraction [32]. No atomic structures of microtubule-bound motor complexes of the kinesin family have been solved, but electron microscopy image reconstruction, using the high-resolution motor and microtubule structures, suggest that kinesin, NCD and KIF1A bind to the α- and β-subunits of tubulin in microtubule [21,33,34]. The binding of the monomeric constructs of kinesin and NCD is similar in the electron micrographs [25,35], and competition experiments indicate their binding sites overlap [36]. The similarity of the motor structures solved thus far [17–19] suggests that other kinesin motors bind similarly.

Cross-linking studies confirm that kinesin and NCD motor domains are proximate to both the α- and β-subunits [37,38]. But it is not known from any of the studies whether the α-subunit involved is intra- or inter-tubulin dimer. The polarity of tubulin is such that the β-subunit in at the GTP-tubulin capped plus end of the microtubule, making the protofilaments $(-)\alpha\beta\alpha\beta\alpha\beta\ldots\alpha\beta\alpha\beta\alpha\beta(+)$. However, the structures of the intramolecular α–β interface and the intermolecular β–α interface are similar [39], in spite of their different stabilities, and cannot be identified unambiguously in high-resolution electron diffraction structures. It is unlikely that the α-subunit contact is lateral to a tubulin in a neigh-

boring protofilament [21]. Whether the binding of a motor domain to the β- and α-tubulin subunits is intra-tubulin or inter-tubulin interface is important. The subunit interactions of a particular motor may be related to its mechanism: for stabilizing or destabilizing microtubules, for determining polarity and processivity, as well as for motor force generation.

We report here investigations of the interactions in solution of *Drosophila* NCD(335–700), a monomeric motor domain construct, with turkey erythrocyte tubulin α,β-dimer (tT). Our original goals were to characterize their interactions and to develop conditions for crystallizing an NCD-tubulin complex, so we used tT which is more homogeneous that bovine brain tubulin [40]. Conditions for crystallization were not found, but our results indicate that under conditions for which tT polymerizes to microtubule, NCD(335–700) increases the rate of polymerization, and that under conditions that do not support polymerization, NCD(335–700) induces polymerization. NCD(335–700) and NCD(335–700) modified with a cysteine-directed fluorescent probe are used to show that for conditions that do not support polymerization, tT binds reversibly and stimulates MgATPase activity of the monomeric motor domain. The results are consistent with NCD(335–700) binding at the interface of two tubulin dimers.

2. Materials and methods

2.1. Protein preparations

Tubulins were isolated from turkey blood (tT) [41], and from bovine brain (bT) [42], purified by phosphocellulose column chromatography [43], flash-frozen in 50 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT pH 6.8, and stored at −80°C until use. This and other buffers were chosen to facilitate NCD binding measurements. Upon thawing, tubulin was stable in this buffer at 0°C for at least 4 h, as assayed by its ability to polymerize at 37°C upon addition of 1 mM GTP and 10% DMSO (v/v). All the measurements reported here for tT were done within 2 h of thawing, except the gel filtration experiments monitoring dimer stability.

NCD(335–700) was prepared as described [44], flash frozen in 25 mM Pipes, 80 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, pH 6.8 and stored at −80°C until use. Upon thawing, NCD(335–700) was stable in this buffer at 0°C for at least 24 h, as assayed by its basal and microtubule-activated MgATPase activity at 10°C.

Protein concentrations were measured by the method of Bradford [45], using myosin subfragment 1 or bovine serum albumin as a standard.

2.2. MgATPase activities

NCD(335–700) MgATPase activities were determined for 1.6 μM NCD(335–700) in 10 mM Mops, 2 mM $MgOAc_2$, 1 mM EGTA, 1 mM DTT, 0.10 mM ATP, at 10°C, either from phosphate production using a malachite green method [46], or from ADP production using a coupled assay [47]. In the coupled assays, a low [ATP] was used to keep above the $K_{M,ATP} = 25$ μM for NCD-mictrotubule [30], but to avoid potential ATP activation of tubulin polymerization. Tubulin or microtubule was added in storage buffer. Addition of comparable volumes of tubulin or microtubule buffer had a negligible effect on NCD activity. Activation of NCD(335–700) activity by tubulin and microtubule was analyzed assuming Michaelis–Menten kinetics to obtain values for V_{MAX} and K_M.

A typical ATPase measurement took 20 min. Control measurements at 10°C were done to ensure that tubulin did not polymerize and microtubule did not depolymerize during the measurement. Turbidity at 400 nm did not change significantly for either sample, indicating negligible changes in polymerization. Centrifugation followed by assay of the supernate for tryptophan fluorescence indicated no changes in polymerization, within experimental error. The presence of NCD may be stabilizing the microtubule at 10°C [16].

2.3. Equilibrium binding

The binding of mians-NCD(335–700) to tubulin

or microtubule at 10°C in 10 mM Mops, 2 mM $MgOAc_2$, 1 mM EGTA, or 1.0 mM Mg-nucleotide, pH 7.0 was detected from steady-state fluorescence intensity changes at 425 nm, while irradiating at 315 nm. Binding curves were determined by serial additions of tT or tMt to NCD(335–700) in solution, or by dilution of concentrated tT-NCD(335–700) samples into NCD(335–700) in solution. Repeated transfers and mixing of NCD(335–700) reduced its fluorescence intensity, and were avoided.

2.4. Tubulin polymerization

Tubulin polymerization was monitored by the increase in the apparent absorbance at 400 nm at increasing times. Polymerization was started by diluting the stock tubulin in 20 mM Pipes, 5 mM $MgOAc_2$, warming to 10 or 37°C and adding 1 mM GTP and 10% DMSO (v/v). For NCD experiments, NCD was added before warming. Addition of NCD storage buffer in the absence of NCD did not change the polymerization detectably. The temperature of the spectrometer cuvette was maintained at 0.5°C.

2.5. Gel filtration chromatography

A medium pressure liquid chromatography system (Bio-Rad Biologic), maintained at 5°C, was used to elute Superdex-200 HR 10/30 column (Pharmacia). Aliquots (100 μl) of 15 μM tT were loaded and the eluent was analyzed for absorbance at 280 nm. Fractions of 0.2 ml were collected for additional analyses. The column volume was eluted within 35 min.

3. Results

3.1. NCD(335-700) stimulation of tubulin polymerization

Routine polymerization of turkey tubulin is complete after 30 min when 8.1 μM t-T is incubated at 37°C in 20 mM Pipes, 5 mM $MgOAc_2$, 1 mM GTP, 10% (v/v) DMSO (Fig. 1). The data are well-fitted using a single exponential function, and the apparent rate constant is $(1.84 \pm 0.03) \times 10^{-3}$ s^{-1}. When 8 μM NCD(335–700) is included in the reaction mixture, the polymerization is

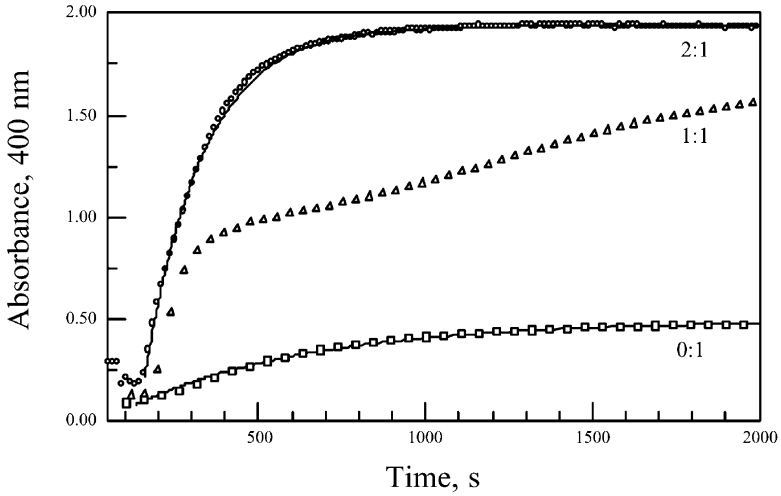

Fig. 1. Turkey tubulin (tT) was polymerized to microtubule at 37°C in the presence of GTP and DMSO. The increase in turbidity was measured, irradiating at 400 nm. In the absence of NCD(335–700) (squares) the apparent first order rate constant is 1.84×10^{-3} s^{-1}, and the data are well-fitted assuming a single exponential reaction. Adding NCD(336–700) increases the rate. When the ratio is 1 NCD(336–700) per tT (triangles), the reaction is biphasic with the rate constant for the faster reaction increased to 5.70×10^{-3} s^{-1}. When the ratio is 2 NCD(336–700) per tT (circles), the faster reaction dominates. Higher [NCD(335–700)] did not increase the rate further.

biphasic, with approximately half of the turbidity increase occurring in a faster phase, followed by a slower phase (Fig. 1). The slower reaction has a rate similar to that observed for tT polymerization in the absence of NCD(335–700). When 16 μM NCD(335–700) is included, all of the tT polymerizes at the fast reaction rate (Fig. 1). Using a single exponential function to fit the data, the fast rate constant in the presence of NCD(335–700) is $5.70 \pm 0.05 \times 10^{-3}$ s^{-1}. Increasing [NCD(335–700)]:[tT] to 2.5 does not further increase the rate of polymerization, within experimental error. The data indicate more than one NCD motor domain binds to a single tubulin, consistent with the reported 2:1 complex of NCD and bovine tubulin [16]. We observed similar increases for polymerization when bT was substituted for tT, but did not investigate the rates or NCD(335–700) concentration dependence (data not shown).

At 10°C, in the polymerization buffer, 8.1 μM tT does not polymerize detectably for at least 45 min (Fig. 2). When 8 μM NCD(335–700) is present, polymerization occurs after a lag period of several minutes (Fig. 2). The lag indicates that the increases observed here and at 37°C above are not due to NCD-tT complex formation, which would occur much more quickly, but are due to polymer formation, consistent with the amplitude of the turbidity increase. Using a single exponential function, the best fit yields $k = 2.8 \pm 0.01 \times 10^{-3}$ s^{-1} for the apparent rate constant for polymerization in the presence of NCD(335–700). The data are statistically somewhat better fit by a two-exponential function, but the second rate constant has a large uncertainty associated with it: $k_1 = 3.4 \pm 0.1 \times 10^{-3}$ s^{-1}, and $k_2 = 3.7 \pm 2.6 \times 10^{-4}$ s^{-1}. The NCD-induced polymerization at 10°C requires GTP, DMSO and the specific interaction of a microtubule-based motor protein. Monomeric kinesin motor domain, K349, also increases the rate of polymerization at 37°C when GTP and DMSO are present. If NCD is replaced by myosin subfragment 1, or if GTP is absent, polymerization does not occur (data not shown).

Aliquots were taken from the 37°C and 10°C reactions and analyzed by transmission electron microscopy after negative staining (not shown). Microtubules were observed in all the cases for which there were increases in turbidity. Microtubules were not observed for samples that did not produce turbidity increases to a stable plateau.

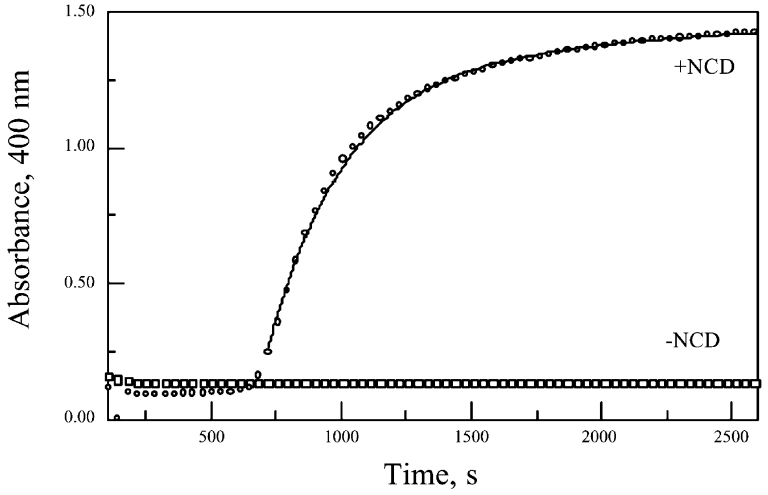

Fig. 2. Turkey tubulin (tT) incubated at 10°C in the polymerization buffer used at 37°C. The turbidity was monitored using irradiation at 400 nm. In the absence of NCD(335–700) (squares), no increase in turbidity was observed, as expected. Adding NCD(335–700) to the solution induced polymerization (circles). When there is 1 NCD(335–700) per tT, the psuedo-first order reaction rate constant is 2.8×10^{-3} s^{-1}.

3.2. Tubulin activation of NCD(335–700) MgATPase activity

The MgATPase activity of NCD(335–700) was 0.0019 ± 0.0002 s^{-1} at 10°C, for 1.6 μM NCD(335–700) in 10 mM MOPS (pH 7.0), 2 mM $MgOAc_2$, 1 mM EGTA, 1 mM DTT, 0.10 mM ATP. Adding tT increased the activity nearly 10-fold (Fig. 3). Assuming that the reaction follows Michaelis–Menten kinetics, K_M is 2.5×10^{-6} M and V_{MAX} is 0.018 s^{-1}. The apparent K_M for tT obtained here is near that reported for bMt activation of NCD MgATPase ($K_M = 3 \times 10^{-6}$ M), but V_{MAX} for tT activation is much lower than for bMt [30]. In the low concentration range, bT activated NCD and mians-NCD MgATPase activity to a similar degree (data not shown).

Under these conditions, in the absence of GTP and DMSO, tT polymerization was not observed in the absence or presence of NCD(335–700) for up to 60 min. Possible Mt formation was assayed by measuring intrinsic tryptophan fluorescence intensity before and after sedimentation at $120\,000 \times g$ to remove any Mt, or by measuring turbidity of the tT-NCD(335–700) solution (see Section 2).

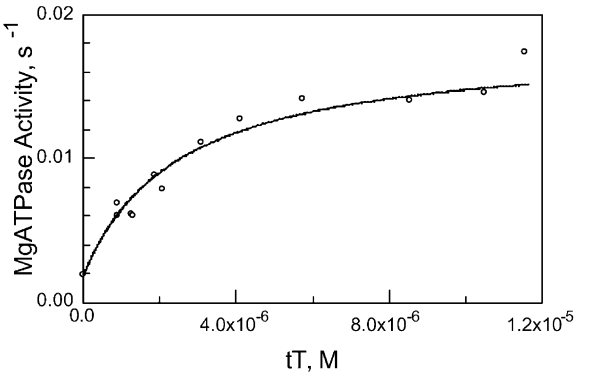

Fig. 3. The rate of MgATP hydrolysis by NCD(335–700) at 10°C is increased from 1.9×10^{-3} s^{-1} to 1.8×10^{-2} s^{-1} (V_{MAX}) by the addition of tT. The Michaelis constant, K_M, is 2.5×10^{-6} M. Under these conditions, with no GTP or DMSO, polymerization of tT is not detectable (see text).

3.3. Synthesis of mians-NCD(335–700)

For experiments to measure the affinity of NCD for tT, NCD(335–700) was covalently modified by 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (mians). Mians (35 μM) was added to 9.5 μM NCD in 10.0 mM MOPS, 2.0 mM $MgOAc_2$, 1.0

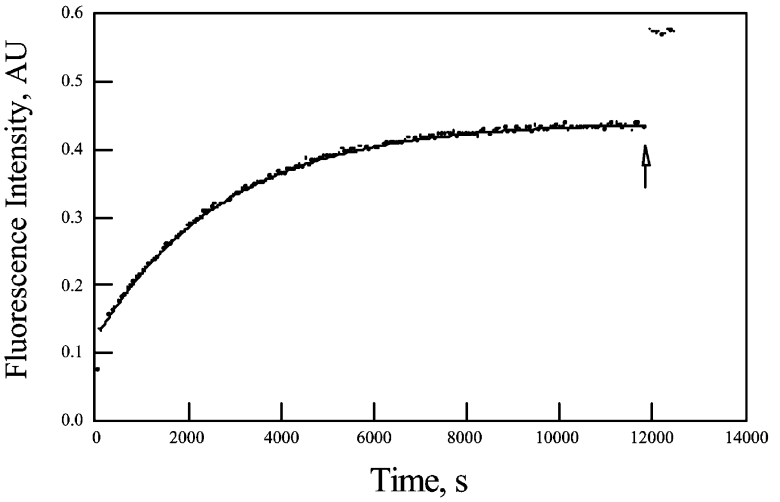

Fig. 4. The time course of the fluorescence intensity increase of the extrinsic probe 2-(4′-maleimidylanilino) naphthalene-6-sulfonic acid (mians) as it reacts to form a covalent attachment to NCD(335–700) is shown. The arrow indicates when β-mercaptoethanol was added to react with excess mians, in order to determine the stoichiometry of the reaction. The kinetic data are well-fitted by a single exponential function, indicating that only one reaction rate is required. The stoichiometry is 0.82 mians per NCD(335–700), suggesting that a single sulfhydryl in being modified.

mM EGTA, pH 7 at 10°C in a quartz cuvette. The solution was irradiated at 315 nm and the emission at 425 nm was monitored until the increase in fluorescence intensity reached a plateau, after approximately 200 min (Fig. 4). The data were fitted using a single exponential function to obtain an apparent rate constant, $k = (3.61 \pm 0.02) \times 10^{-4}$ s^{-1}.

An aliquot of excess β-mercaptoethanol (β-ME) was then added to the cuvette to react with any remaining mians. The concentration of unreacted mians was determined from the increase in the fluorescence intensity due to reaction with β-ME, by comparison to increases for known amounts of mians-β-ME. The concentration of mians that reacted with NCD(335–700) was taken as the difference of the total mians and the amount that reacted with β-ME. Inner filter effects were ignored. The stoichiometry of labeling was determined to be 0.82:1 for mians:NCD(335–700). This stoichiometry is close to that observed for modification of NCD with a maleimide spin label [48]. The maleimide fluorescence label, used here, probably modified the same hyperactive residue, cys-670, reported by others [16,48]. The basal MgATPase activity of mians-NCD(335–700) is increased 20 and 40%, at 10 and 37°C, respectively, compared to unmodified NCD(335–700). Adding equimolar tMt to 1.3 μM NCD(335–700) and mians-NCD(335–700) increased the MgATPase activity 2.5- and 2.1-fold, respectively, compared to the basal activities. The MgATPase activity of mians-NCD(335–700) is also activated by tT, approximately to the same degree as unlabeled NCD(335–700). Although there are too few data to determine K_M or V_{MAX}, the results indicate that NCD(335–700) and mians-NCD(335–700) interact with tT and tMt similarly.

For routine use, frozen purified NCD(335–700) was thawed, diluted to 66 μM in 10.0 mM MOPS, 2.0 mM $MgOAc_2$, 1.0 mM EGTA pH 7 at 0°C and 52 μM mians was added. The solution was incubated on ice overnight, and used without further purification. Addition of β-ME to mians-NCD(335–700) prepared this way did not increase the fluorescence intensity significantly, indicating that unreacted mians was negligible.

3.4. Tubulin binding to mians-NCD(335–700)

The fluorescence intensity of mians-NCD(355–700) in the presence of excess MgADP, at 10°C, increases when tT is added to the solution (Fig. 5). Assuming that the stoichiometry of binding is 2:1, the data are best fit if $K_D = 1.1 \times 10^{-5}$ M and the fluorescence intensity of the tT.mians-NCD(335–700) complex is 1.97-fold that of free mians-NCD(335–700). Replacing ADP with AMPPNP or ATP has little effect on K_D. We could not detect significant tubulin polymerization during the time required to measure binding, by monitoring turbidity or tryptophan fluorescence intensity after centrifugation to remove microtubule from solution. Changes as small as 3% would have been detected.

Results for turkey microtubule (tMt) were compared to those for tT. When tT is polymerized to tMt at 37°C as described above, and then used at 10°C, the apparent K_D decreases to 0.99×10^{-6} M for binding to mians-NCD(335–770) in the presence of MgADP (Fig. 6). The K_D at comparable ionic strength for binding to bovine brain microtubule of monomeric NCD has been reported as 0.20×10^{-6} M at 25°C [28,49] and 0.68×10^{-6} M at 20°C [16], consistent with our result. No tMt stabilizing agents were used in the present experiments, so tMt depolymerization could make this apparent K_D higher than the true

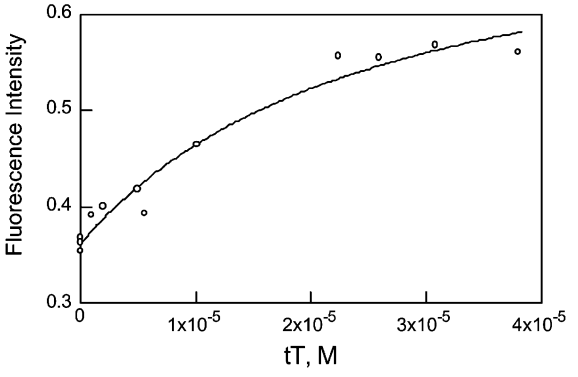

Fig. 5. The dissociation constant for the binding of mians-NCD(335–700) to tT was determined to be 2.3×10^{-5} M at 10°C, from the [tT] dependence of the 97% increase in fluorescence intensity.

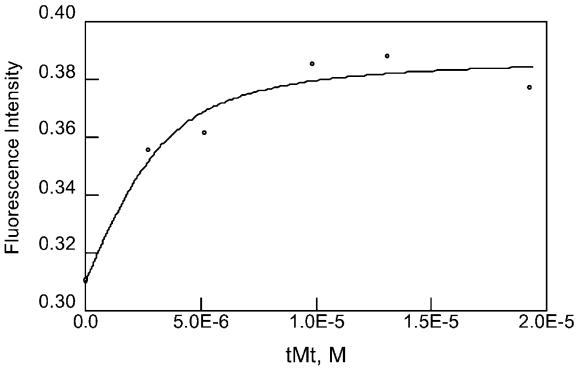

Fig. 6. The dissociation constant for the binding of mians-NCD(335–700) to tMt was determined to be 9.9×10^{-7} M at 10°C, from the observed [tMt] dependence of the 44% increase in fluorescence intensity. Concentrated tMt was formed at 37°C and diluted into the mians-NCD(335–700) solution. Depolymerization was not detectable (see text).

value. However, in the absence of mians-NCD(335–700) no depolymerization was detectable during the 1-h required to measure binding. Depolymerization is less likely to occur in the presence of mians-NCD(335–700) [16]. No corrections were made for the residual MgGTP and DMSO present in the solutions used to measure the affinity of tMt to mians-NCD(335–770), which were not present in the tT binding measurements. The fluorescence intensity of the tMt-mians-NCD(335–700) complex is 1.44 times that of free mians-NCD(335–700).

3.5. tT stability

It is crucial for interpretation of many of the experiments above that dimeric tT be the molecular species that is causing the observed signal. To test for the presence of microtubules, aliquots were taken from the tT solutions before and after high-speed centrifugation and the intrinsic tryptophan fluorescence intensity was measured. At 10°C, during the 2 h after thawing used to make the above measurements, the fluorescence intensity of tT solutions was not decreased detectable by centrifugation at $120\,000 \times g$ for 20 or 60 min. These experiments indicate that microtubule formation is negligible for the conditions used. After polymerization, the fluorescence intensity decreased to less than 3% by the same procedure, indicating polymerization was complete, within experimental error.

Smaller aggregates of tubulin, which would be expected to sediment at rates not much greater than that of dimeric tubulin cannot be resolved by our centrifugation method. To assay for these oligomeric species, tT solutions were incubated at 10°C in the buffer used to measure binding to miansNCD(335–700) and aliquots were taken at increasing times and eluted on a Pharmacia Superdex 200 HR 10/30 column. Only three peaks are detected by absorbance at 280 nm (Fig. 7a), a major peak with an elution time consistent with the molecular weight of dimeric tubulin, a minor peak with an elution time consistent with nucleotide or other molecules too small to be protein, and a minor peak for an unknown larger species ($M_r > 10^6$ Da) that is eluted in the exclusion volume. The major tT dimer peak accounts for over 95% of the total absorbance during the 2 h after thawing, during which the polymerization and binding experiments were done. At longer times, the minor peaks associated with the larger particle and with GDP increase significantly in size, and the tT dimer peak decreases (Fig. 7b,c).

The apparent higher molecular weight species presents a potential problem if it interacts with NCD(335–700). To determine some of the properties of this material, a tT solution was incubated at 0°C for 16 h, and the high molecular weight species was collected. It is not removed from solution by ultracentrifugation, indicating that it is not microtubule. Using the fluorescence method described above for tT and tMt binding to mians-NCD(335–700), binding of the apparent high molecular weight material could not be detected. In contrast, material from the tT peak, isolated after 16 h incubation, gives the same fluorescence increase per mole, when mixed with mians-NCD(335–700) as does freshly thawed tT preparation.

These experiments indicate that the tT dimer is stable and homogeneous for at least 2 h after

thawing. Microtubule formation under the conditions used to investigate tT is undetectable. Some non-microtubule higher molecular weight species are being formed steadily from tT, but this material does not bind mians-NCD(335–700), and probably does not bind NCD.

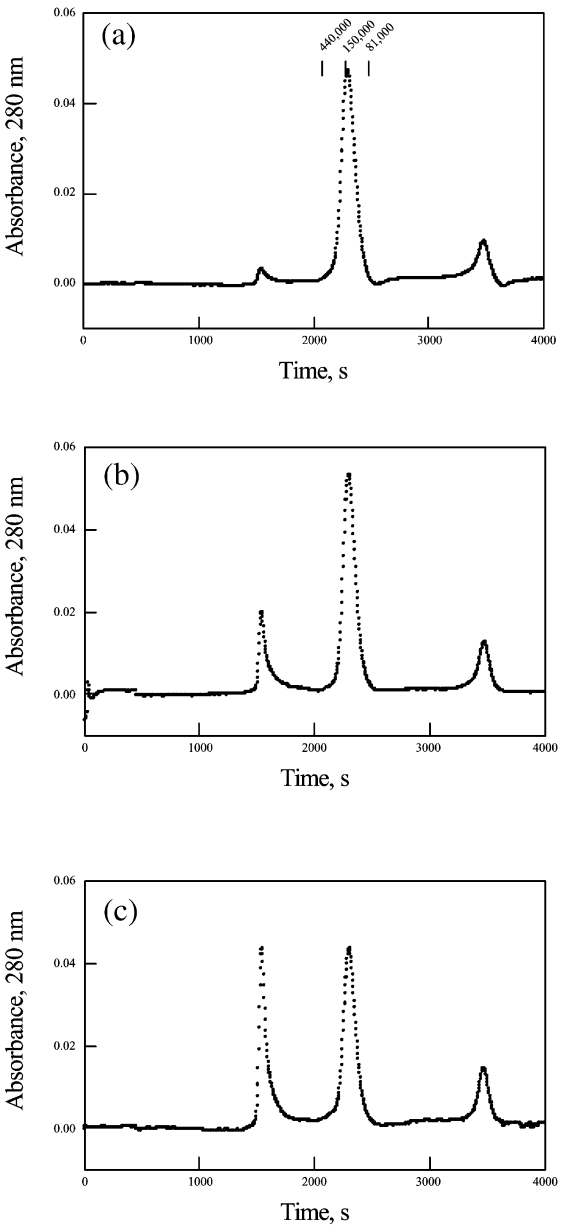

Fig. 7.

4. Discussion

The effect of NCD(335–700) on tT polymerization is striking. At 37°C, under standard conditions that promote polymerization, it increases the rate threefold (Fig. 1). At 10°C, under conditions that promote depolymerization, NCD(335–700) induces polymerization, which proceeds at a rate comparable to rates observed at 37°C (Fig. 2). In both cases, after the increase in turbidity, the microtubules are removed by centrifugation and are observable in electron micrographs. GTP is required for the NCD(335–700) activation. The quantitative effect at 37°C and the qualitative change in behavior at 10°C suggest NCD motor domain interacts with tubulin dimers to induce polymerization.

When GTP is absent, polymerization in the presence of NCD(335–700) at 10°C is not observed, although other experiments reported here indicate that NCD(335–700) and tT interact. The activation at 10°C of NCD(335–700) MgATPase by tT (Fig. 3) and the increase in mians-NCD(335–700) fluorescence intensity when tT binds (Fig. 5) occur with no detectable tT polymerization. Taken together, these data are consistent with NCD(335–700) promoting tT polymerization by decreasing the activation energy of the rate determining step on the normal tubulin polymerization pathway.

The [NCD(335–700)] dependence of the rate enhancement of tT polymerization at 37°C indi-

Fig. 7 A freshly prepared sample of tT in the buffer used for the binding assay was incubated at 0°C and aliquots were taken at increasing times and analyzed by gel filtration chromatography at 4°C using a Superdex-200 HR 10/30 column. Three peaks were observed: an early peak corresponding to material hydrodynamically larger that tT α,β-dimer; a middle peak corresponding to α,β-dimer; and a third peak corresponding to GDP and other very low molecular weight species. Panels a, b and c show elution patterns for aliquots taken at 0, 625 and 1660 min, respectively, after thawing a tT sample. The material in the first peak does not sediment under conditions that remove microtubule, and does not bind to mians-NCD(335–700). The elution times of molecular weight standards, ferritin (M_r 440 000), human IgG (M_r 150 000) and human transferrin (M_r 81 000) are shown for comparison in panel a.

cates that more than one NCD(335–700) molecule interacts with tT (Fig. 1). This observation is consistent with results reported for NCD interactions with bT, which show that two NCD molecules can bind to bT [16]. In the same study it was shown that bT activates NCD MgATPase approximately sixfold, consistent with the 10-fold enhancement by tT reported here. On the other hand, some of the results for bT and tT do not appear to be consistent. Specifically, NCD binding to bT, in the presence of MgADP in low ionic strength buffer (~ 0.02 M) is fourfold stronger than binding to bMt [16], while for similar conditions NCD(335–700) binding to tT is 10-fold weaker than binding to tMt (Figs. 6 and 7). The reason for this difference is not known. The affinities for bMt and tMt are approximately equal (Fig. 6 [16]).

The other apparent inconsistency is that NCD does not induce bT polymerization [16]. It was suggested that a second NCD binding site on tubulin becomes inaccessible once polymerization occurs, and concluded that the NCD-bT complex is 'almost totally inactive' [16]. The tT and bT sequences are nearly identical. It is doubtful that the difference in activation of polymerization is due to the differences in glycosylation of bT and tT [40], or in NCD cysteine modification. It seems more likely that the differences in the observations and conclusions for bT and tT are due to the absence or presence of GTP. We observe stable non-reactive NCD-tT complexes when GTP is absent (Figs. 4 and 5), consistent with the bT results [16], but polymerization enhancement when GTP is present (Figs. 1 and 2). Taken together, the results suggest that NCD binds tubulin, and if GTP is present, promotes polymerization. The need for GTP is consistent with its role in the GTP-tubulin cap that enhances Mt elongation and protects against catastrophic depolymerization [22,50,51].

There are two alternative categorical pathways by which NCD(335–700) can accelerate tT polymerization. In one category are mechanisms in which NCD(335–700) activates tubulin: it binds to tT dimer forming a complex, NCD(335–700)$_m$.tT ($m \geq 1$), which nucleates and/or adds to Mt more rapidly than does tT itself. In the other category are mechanisms in which NCD(335–700) stabilizes existing tubulin aggregates: n molecules of tT aggregate to form tT$_n$, followed by NCD(335–700) binding to form NCD(335–700).tT$_n$, which elongates more rapidly, or dissociates more slowly, than does tT$_n$ itself. In all cases, the final product is normal microtubule that has one NCD(335–700) bound per tubulin dimer subunit [33].

We cannot choose between these two possibilities with certainty. At 10°C tubulin does not polymerize, and microtubules depolymerize, even in the presence of GTP [52], suggesting that no aggregates of tT would be present to bind NCD(335–700) and produce the polymerization observed at 10°C (Fig. 2). Consistent with this observation, the activation of NCD(335–700) MgATPase activity by tT at 10°C occurs with no detectable formation of microtubule or other aggregated species. These data suggest that enhanced polymerization and MgATPase activity are due to tT dimer binding NCD(335–700). The apparent stoichiometry of $\sim$ 2:1 for NCD(335–700):tT in the polymerization reaction in the presence of GTP at 37°C also favors formation of an activated NCD-tT complex, rather than stabilization of aggregated tT. NCD(335–700)$_2$.tT complexes are structurally reasonable, while complexes such as NCD(335–700)$_{2n}$. tT$_n$ are less plausible, if they are axial extensions. Laterally associated complexes made up of more than one NCD(335–700)$_2$.tT cannot be excluded. The 2:1 complex suggested here as a reactive intermediate in the presence of GTP is consistent with bT binding two NCD monomers when GTP is absent [16]. The hypothesis that NCD(335–700)$_2$.tT is an activated species involved in polymerization also is consistent with the observation that tubulin dimer undergoes a conformational change to a more reactive species before it aggregates [53–55]. This conformational change is followed by GTP hydrolysis [56], which occurs in microtubule tubulin subunits below the GTP cap [24,51].

NCD(335–700) binding to form NCD(335–700)$_2$.tT can reduce the activation energy for polymerization in two ways. Firstly, by binding to tT, NCD(335–700) can lower the activation energy by changing the tT conformation.

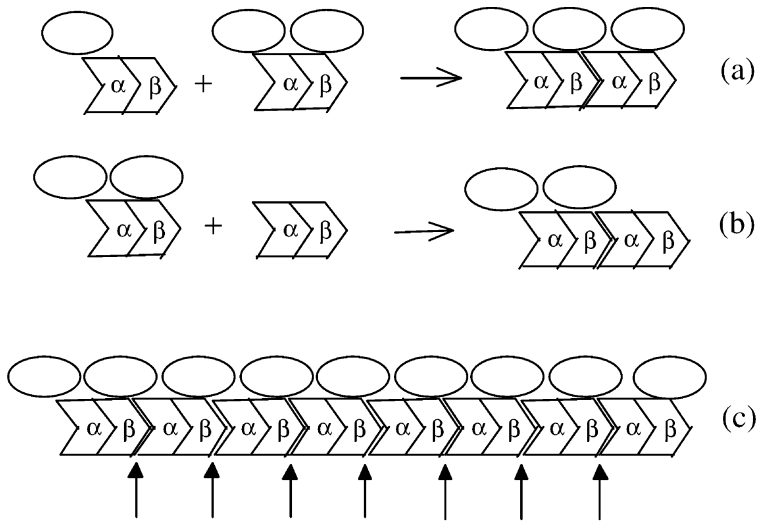

Fig. 8. A schematic model and reactions for NCD(335–700) activation of tubulin–tubulin interaction. Two NCD(335–700) (oval shape) can bind one tT (chevron shape). Binding may change the conformation of tT to make it more reactive. The NCD(335–700)$_2$.tT complex also has unfilled tT binding subsites on each NCD(335–700) molecule (see a and b). When a tT binding subsite is occupied by NCD(335–700).tT or tT, the tubulins are positioned in a way that promotes initiation and/or polymerization, in the presence of GTP. The specific geometry shown is arbitrary; the second tT could bind behind, in front of, or next to the tT in the NCD(335–700)$_2$.tT complex. The reactions suggest initiation, but apply to polymerization as well. After polymerization (c), NCD:tubulin is 1:1, with each NCD motor domain binding at the interface (arrows) of α- and β-subunits on different tubulin dimers.

Secondly, if NCD(335–700)$_2$.tT is a reactive intermediate it can provide an additional tT binding subsite, which when occupied by tT or NCD(335–700).tT, positions a second tT in such a way as to promote tT-tT interaction (see Fig. 8). This mechanism may be similar to the initiation of tubulin self-assembly, which involves positioning of tubulin dimers to promote longitudinal [57] or lateral [58] interactions. It is not being suggested that NCD(335–700) is necessarily involved in in vivo initiation of Mt self-assembly, but that complexes such as NCD(335–700)$_2$.tT (Fig. 8) have the potential to promote tubulin polymerization in vitro by enthalpic and entropic contributions to lowering the activation energy. However, the results may be related to in vivo NCD motor function. Image analysis of NCD-decorated microtubules indicate that the ratio of motor domain to tubulin dimer is 1:1, and that one monomeric motor domain makes contact with two tubulin monomers in a single protofilament [33]. The data reported here help to clarify an ambiguity in the field and suggest that a NCD motor domain binds to a microtubule at the interface of α and β subunits of neighboring tubulin dimers, rather than at the interface of subunits of the same tubulin.

Acknowledgements

We thank Nariman Naber and Sarah Rice, for the generous gifts of bovine tubulin and K349(C220), respectively, Rebeka Barth for making some preliminary fluorescence measurements, and Barbara Plowman for the electron microscopy of the microtubules. S.H. also thanks J.J. Correia for a helpful discussion of tubulin stability. Supported by NIH grant AR42895.

References

[1] D.J. Komma, A.S. Horne, S.A. Endow, Separation of meiotic and mitotic effects of claret non-disjunctional

on chromosome segregation in Drosophila, EMBO J. 10 (1991) 419–424.
[2] H.J.G. Matthies, H.B. McDonald, L.S.B. Goldstein, W.E. Theurkauf, Anastral meiotic spindle morphogenesis: Role of the non-claret disjunctional kinesin-like protein, J. Cell Biol. 134 (1996) 455–464.
[3] R. Chandra, E.D. Salmon, H.P. Erickson, A. Lockhart, S.A. Endow, Structural and functional domains of the Drosophila ncd microtubule motor protein, J. Biol. Chem. 268 (1993) 9005–9013.
[4] R.A. Walker, E.D. Salmon, S.A. Endow, The Drosophila claret segregation protein is a minus-ended directed motor molecule, Nature 347 (1990) 780–782.
[5] H.B. McDonald, R.J. Stewart, L.S.B. Goldstein, The kinesin-like ncd protein of Drosophila is a minus end-directed microtubule motor, Cell 63 (1990) 1159–1165.
[6] R.B. Case, D.W. Pierce, N. Hombooher, C.L. Hart, R.D. Vale, The directional preference of kinesin motors is specified by an element outside of the motor catalytic domain, Cell 90 (1997) 959–966.
[7] I.M.T.C. Crevel, A. Lockhart, R.A. Cross, Kinetic evidence for low chemical processivity in ncd and Eg5, J. Mol. Biol. 273 (1997) 160–170.
[8] K.A. Foster, S.P. Gilbert, Kinetic studies of dimeric Ncd: Evidence that Ncd is not processive, Biochemistry 39 (2000) 1784–1791.
[9] A. Karabay, R.A. Walker, The Ncd tail domain promotes microtubule assembly and stability, Biochem. Biophys. Res. Commun. 258 (1999) 39–43.
[10] R.D. Vale, R.A. Milligan, The way things move: Looking under the hood of molecular motor proteins, Science 288 (2000) 88–95.
[11] R.D. Vale, T. Reese, M.P. Sheetz, Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility, Cell 42 (1985) 39–50.
[12] C.E. Walczak, T.J. Mitchison, A. Desai, XKCM1: A Xenopus kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly, Cell 84 (1996) 37–47.
[13] A. Desai, S. Verma, T.J. Mitchison, C.E. Walczak, Kin I kinesins are microtubule-destabilizing enzymes, Cell 96 (1999) 69–78.
[14] S.A. Endow, S.J. Kang, L.L. Satterwhite, M.D. Rose, V.P. Skeen, E.D. Salmon, Yeast Kar3 is a minus-end microtubule motor protein that destabilizes microtubules preferentially at the minus ends, EMBO J. 13 (1994) 2708–2713.
[15] J.G. Barrett, B.D. Manning, M. Snyder, The Kar3p kinesin-related protein forms a novel heterodimeric structure with its associated protein Cik1p, Molec. Biol. Cell 11 (2000) 2373–2385.
[16] C. Smyczynski, J. Derancourt, P. Chaussepied, Regulation of ncd by the oligomeric state of tubulin, J. Mol. Biol. 295 (2000) 325–336.
[17] F.J. Kull, E.P. Sablin, R. Lau, R.J. Fletterick, R.D. Vale, Crystal structure of the kinesin motor domain reveals a structural similarity to myosin, Nature 380 (1996) 550–555.
[18] E.P. Sablin, F.J. Kull, R. Cooke, R.D. Vale, R.J. Fletterick, Crystal structure of the motor domain of the kinesin-related motor ncd, Nature 380 (1996) 555–559.
[19] A.M. Gulick, H. Song, S.A. Endow, I. Rayment, X-Ray crystal structure of the yeast Kar3 motor domain complexed with Mg·ADP to 2.3 angstrom resolution, Biochemistry 37 (1998) 1769–1776.
[20] M. Kikkawa, Y. Okada, N. Hirokawa, 15 Angstrom resolution model of the monomeric kinesin motor, KIF1A, Cell 100 (2000) 241–252.
[21] M. Kikkawa, E.P. Sablin, Y. Okada, H. Yajima, R.J. Fletterick, N. Hirokawa, Switch-based mechanism of kinesin motors, Nature 411 (2001) 439–445.
[22] T. Mitchison, M. Kirschner, Microtubule assembly nucleated by isolated centrosomes, Nature 312 (1984) 232–237.
[23] M. Caplow, J. Shanks, Evidence that a single monolayer tubulin-GTP cap is both necessary and sufficient to stabilize microtubules, Molec. Biol. Cell 7 (1996) 663–675.
[24] H.P. Erickson, E.T. O'Brien, Mirotubule dynamic instability and GTP hydrolysis, Annu. Rev. Biophys. Biomolec. Struct. 21 (1992) 145–166.
[25] A. Hoenger, E.P. Sablin, R.D. Vale, R.J. Fletterick, R.A. Milligan, Three-dimensional structure of a tubulin-motor-protein complex, Nature 376 (1995) 271–274.
[26] K. Hirose, R.A. Cross, L.A. Amos, Nucleotide-dependent structural changes in dimeric NCD molecules complexed to microtubules, J. Mol. Biol. 278 (1998) 389–400.
[27] A. Hoenger, S. Sack, M. Thormahlen et al., Image reconstructions of microtubules decorated with monomeric and dimeric kinesins: Comparison with X-ray structure and implications for motility, J. Cell Biol. 141 (1998) 419–430.
[28] K.A. Foster, J.J. Correia, S.P. Gilbert, Equilibrium binding studies of non-claret disjunctional protein (Ncd) reveal cooperative interactions between the motor domains, J. Biol. Chem. 273 (1998) 35307–35318.
[29] T. Shimizu, E. Sablin, R.D. Vale, R. Fletterick, E. Pechatnikova, E.W. Taylor, Expression, purification, ATPase properties, and microtubule-binding properties of the ncd motor domain, Biochemistry 34 (1995) 13259–13266.
[30] E. Pechatnikova, E.W. Taylor, Kinetic mechanism of monomeric non-claret disjunctional protein (Ncd) ATPase, J. Biol. Chem. 272 (1997) 30735–30740.
[31] E. Pechatnikova, E.W. Taylor, Kinetics processivity and the direction of motion of Ncd, Biophys. J. 77 (1999) 1003–1016.
[32] E. Nogales, M. Whittaker, R.A. Milligan, K.H. Downing, High-resolution model of the microtubule, Cell 96 (1999) 79–88.
[33] H. Sosa, R.A. Milligan, Three-dimensional structure of ncd-decorated microtubules obtained by a back-projection method, J. Mol. Biol. 260 (1996) 743–755.

[34] E. Mandelkow, E.M. Mandelkow, Microtubule structure, Curr. Opin. Struct. Biol. 4 (1994) 171–179.
[35] M. Kikkawa, T. Ishikawa, T. Wakabayashi, N. Hirokawa, Three-dimensional structure of the kinesin head-microtubule complex, Nature 376 (1995) 274–277.
[36] H. Song, S.A. Endow, Binding sites on microtubules of kinesin motors of the same or opposite polarity, Biochemistry 35 (1996) 11203–11209.
[37] R.A. Walker, ncd and kinesin motor domains interact with both alpha- and beta-tubulin, Proc. Natl. Acad. Sci. USA 92 (1995) 5960–5964.
[38] C. Tucker, L.S.B. Goldstein, Probing the kinesin-microtubule interaction, J. Biol. Chem. 272 (1997) 9481–9488.
[39] E. Nogales, Structural insights into microtubule function, Annu. Rev. Biochem. 69 (2000) 277–302.
[40] M. Rudiger, K. Weber, Characterization of the post-translational modifications in tubulin from the marginal band of avian erythrocytes, Eur. J. Biochem. 218 (1993) 9681–9689.
[41] D.B. Murphy, Purification of tubulin and tau from chicken erythrocytes: Tubulin isotypes and mechanisms of microtubule assembly, Meth. Enzymol. 196 (1991) 235–246.
[42] A.A. Hyman, S. Salser, D.N. Drechsel, N. Unwin, T.J. Mitchison, Role of GTP hydrolysis in microtubule dynamics: information from a slowly hydrolyzable analogue, GMPCPP, Molec. Biol. Cell 3 (1992) 1155–1167.
[43] R.C. Williams, J.C. Lee, Preparation of tubulin from brain, Meth. Enzymol. 85 (1982) 376–385.
[44] E.P. Sablin, R.J. Fletterick, Crystallization and preliminary structural studies of the ncd motor domain, Prot.-Struct. Funct. Genet. 21 (1995) 68–69.
[45] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[46] T. Kodama, K. Fukui, K. Kometani, The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 studied by a modified malachite green method for determination of inorganic phosphate, J. Biochem. (Tokyo) 99 (1986) 1465–1472.
[47] K. Imamura, M. Tada, Y. Tonomura, Pre-steady state of the myosin-adenosine triphosphate system. IV. Liberation of ADP from the myosin-ATP system and effects of modifiers on the phosphorylation of myosin, J. Biochem. (Tokyo) 59 (1966) 280–289.
[48] N. Naber, R. Cooke, E. Pate, Binding of ncd to microtubules induces a conformational change near the junction of the motor domain with the neck, Biochemistry 36 (1997) 9681–9689.
[49] A.T. Mackey, S.P. Gilbert, Moving a microtubule may require two heads: A kinetic investigation of monomeric Ncd, Biochemistry 39 (2000) 1346–1355.
[50] T.A. Walker, E.T. O'Brien, N.K. Pryer et al., Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies, J. Cell Biol. 107 (1988) 1437–1448.
[51] A. Desai, T.J. Mitchison, Microtubule polymerization dynamics, Annu. Rev. Cell Dev. Biol. 13 (1997) 83–117.
[52] T. Mitchison, M. Kirschner, Dynamic Instability of microtubule growth, Nature 312 (1984) 237–242.
[53] M.-F. Carlier, Kinetic evidence for a conformation change of tubulin preceding microtubule assembly, J. Biol. Chem. 258 (1983) 2415–2420.
[54] M.-F. Carlier, D. Didry, D. Pantaloni, Hydrolysis of GTP associated with the formation of tubulin oligomers is involved in microtubule nucleation, Biophys. J. 73 (1997) 418–427.
[55] W.A. Voter, H.P. Erickson, The kinetics of microtubule assembly. Evidence for a two-stage nucleation mechanism, J. Biol. Chem. 259 (1984) 10430–10438.
[56] T. David-Pfeuty, H.P. Erickson, D. Pantaloni, Guanosinetriphophatase activity of tubulin associated with microtubule assembly, Proc. Natl. Acad. Sci. USA 74 (1977) 5372–5376.
[57] Y. Zheng, M.L. Wong, B. Alberts, T. Mitchison, Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex, Nature 378 (1995) 578–583.
[58] H.P. Erickson, D. Stoffler, Protofilaments and rings, two conformations of the tubulin family conserved from bacterial FtsZ to alpha/beta and gamma tubulin, J. Cell. Biol. 135 (1996) 5–8.