Effect of Tubulin Self-Association on GTP Hydrolysis and Nucleotide Exchange Reactions

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GTP Hydrolysis and Nucleotide Exchange

Abstract

Tubulin nucleation, microtubule assembly, stability, and dynamics depend on fluxes of chemical energy, controlled by hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP), and nucleotide exchange reactions. In this paper, we determined how tubulin self-association in glycerol-free assembly buffers affects the rate of GTP hydrolysis and the thermodynamics of nucleotide exchange. In the absence of tubulin, GTP hydrolysis was negligible. In the presence of tubulin, below the critical conditions for microtubule assembly, no GTP hydrolysis was observed, even though tubulin 1D curved oligomers and single rings were formed, suggesting that GTP hydrolysis was not involved in their formation. Under conditions permitting spontaneous tubulin nucleation and microtubule assembly, GTP hydrolysis was detected and followed pseudo first-order kinetics, limited by the rate of tubulin nucleation and microtubule assembly. By simultaneously determining the concentrations of tubulin-free and tubulin-bound GTP and GDP at steady-state, we investigated the nucleotide exchange reaction under conditions where GTP hydrolysis was negligible. The exchange reaction strongly depended on the molar ratio between tubulin-free GDP and GTP and the total tubulin concentration. To analyze these data, we used a thermodynamic model of isodesmic tubulin self-association, terminated by the formation of tubulin single-rings, to calculate the distribution of tubulin single rings, 1D oligomers, and free dimers, and thereby the molar fractions of tubulin dimers with exposed and buried nucleotide exchangeable sites (E-sites). Our data suggest that the exchange reaction occurred to a different extent on tubulin dimers with buried E-sites than on dimers with exposed E-sites.

Abbreviations

Microtubule, MT; guanosine triphosphate, GTP; guanosine diphosphate, GDP; guanosine monophosphate, GMP; adenosine triphosphate, ATP; High performance liquid chromatography, HPLC; microtubule associated proteins, MAPs

Introduction

Microtubules (MTs) are dynamic biopolymers, self-assembled from $\alpha\beta$ -tubulin heterodimer subunits. MTs are essential components of several cellular architectures including the cytoskeleton and the mitotic spindle.^{1,2} MT dynamics, allowing assembly and disassembly of these structures, depends on a plethora of parameters including, post-translational modifications,³ ionic composition and strength,⁴⁻⁶ temperature,⁵ additives and drugs,⁵ and MT-associated proteins (MAPs).^{7,8}

Two nucleotides are attached to each tubulin dimer, one at a non-exchangeable site (N-site), located on the α subunit at the interface between the subunits, and one at an exchangeable site (E-site), located on the β subunit.^{9,10} The N-site nucleotide is predominantly guanosine triphosphate (GTP), whereas, at the E-site, the nucleotide can be either guanosine diphosphate (GDP) or GTP. In curved assemblies of GDP-tubulin, the E-site nucleotide is not directly exposed to the solvent (Figure 1).¹¹

MTs nucleate and elongate mainly from tubulin dimers with GTP at their E-site, (GTP-tubulin or tub-GTP).¹⁶⁻¹⁸ GTP hydrolysis to GDP-tubulin (tub-GDP) at the tubulin E-site, occurs during MT assembly following either tubulin nucleation (leading to the creation of a new MT) or MT elongation.^{8,19-24} The kinetics of GTP hydrolysis is critical for MT stability and dynamics.^{1,8,18} The hydrolysis is tightly associated with structural changes in the MT lattice, leading to more compact dimers, and weakening the lateral interaction between dimer subunits.^{1,22,24-31} Recently, it was suggested that GTP hydrolysis is increasing the longitudinal lattice tension, contributing to more confined and correlated GDP-tubulin dynamics, thereby entropically destabilizing the MT lattice.³²⁻³⁴ Hydrolysis interferes with tubulin nucleation and MT assembly^{17,18,29,35-37} and may affect the affinity of MAPs to the MT lattice.²² The molar ratio between GTP- and GDP-tubulin is constantly changing during the hydrolysis reaction. GTP-hydrolysis occurs at the interface between the GTP-tubulin cap and the GDP-tubulin MT core,²³ and may be slower than the rate of subunit addition.^{19,38} The delay is associated with structural rearrangements of the tubulin on the

Figure 1: The interface between α - and β -tubulin in a curved 1D GDP-tubulin assembly, leading to tubulin single ring symmetry.¹² The atomic tubulin model is based on PDB ID 5JQG.¹¹ Missing residues were added and refined using Modeller,¹³ as explained.¹⁴ The cartoon was created by PyMOL.¹⁵ β - and α -tubulin are colored red and green, respectively. The GDP molecule, occupying the E-site on the β -tubulin, is represented by cyan spheres, whereas the GTP molecule occupying the N-site on the α -tubulin, is represented by orange spheres. The first tubulin dimer in the assembly, for which the β monomer is shown (red), is in a *Buried* conformation.

MT lattice, required for GTP hydrolysis.^{22,36}

In addition to GTP hydrolysis, a tubulin-bound nucleotide can be modified by the following exchange reaction with an unbound (free) nucleotide

tub-GTP can facilitate MT assembly, hence nucleotide exchange is a fundamental process in MT dynamics.¹⁸ The extent of exchange varies with solution conditions such as nucleotide composition and concentration.³⁹ When Mg^{2+} is added, it can form complexes with the nucleotides, the tubulin or both, and several reversible equilibrium reactions can take place.⁴⁰ The molar ratio between GTP- and GDP-tubulin controls the nucleation of tubulin and the stability of preassembled MTs.¹⁶ Knowing its value is therefore crucial for analyzing tubulin nucleation and assembly reactions.⁴¹

In this paper, we determined the manner by which tubulin self-association in glycerol-free assembly buffers affects the rate of GTP hydrolysis and the thermodynamics of nucleotide exchange. We measured the rates of GTP hydrolysis in the absence and presence of tubulin, at 4 and 36 °C. In the absence of tubulin, marginal GTP hydrolysis was observed at 36 °C. In the presence of tubulin, no GTP hydrolysis was observed below the critical conditions for microtubule assembly, even though tubulin 1D curved oligomers and single rings were formed,^{12,14} suggesting that GTP hydrolysis was not involved in their formation. GTP hydrolysis was observed under conditions permitting spontaneous MT assembly and the hydrolysis followed a pseudo first-order kinetics, limited by the rate of tubulin nucleation and MT elongation.

The GDP to GTP exchange reaction mechanism on tubulin was studied under conditions of negligible GTP hydrolysis at different GTP to GDP molar ratios and tubulin concentrations. By simultaneously measuring the tubulin-free and tubulin-bound nucleotide composition, we found that the reaction mechanism should take into account the self-association

of tubulin, leading to formation of tubulin dimers with exposed and buried E-sites.^{42,43} Our data also suggest that the association of Mg^{2+} ions with the tubulin dimers was primarily via their association with the free nucleotides.

Materials and Methods

Materials were purchased from Sigma-Aldrich Co. Israel. The precision of our temperature measurements was $\pm 1^\circ\text{C}$.

Tubulin Purification

Tubulin was purified from porcine brains, using a modified version of the high-molarity buffer purification method,⁴⁴ described¹² and used in our earlier publications.^{6,45–47} The tubulin assembly buffer was BRB80 (80 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM MgCl_2) at pH 6.9 (KOH) and typically contained 0.4 ± 0.1 mM adenosine triphosphate (ATP), 0.5 ± 0.1 mM GTP, and 0.4 ± 0.1 mM GDP. After the purification, aliquots of 36 ± 2 mg/mL tubulin were flash-frozen in liquid nitrogen and stored at -80°C . Before used, tubulin solutions were thawed on ice.

Sample Preparation Protocols

Incubation Protocol

Tubulin samples for steady-state experiments, at different GDP to GTP molar ratios, obtained by either nucleotide addition or the Buffer Exchange protocol, were incubated for about 2 h at the experimental temperature. The samples were then analyzed by the Nucleotide Extraction by Protein Precipitation protocol.

Heat-Cool Cycles

GDP-tubulin was also obtained through hydrolysis of GTP by applying seven heat-cool cycles to the tubulin solutions, as explained.¹² A heat-cool cycle included a 35 min incubation at 36 °C followed by a 40 min incubation at 5 °C. After the last cycle, the samples were spun at 4 °C and 20800 g for 30 min. The top 60% of the sample volume was used for HPLC Analysis.

SEC Protocol

500 μ L of ice-cold 36 ± 2 mg/mL tubulin solution was injected into a SuperdexTM 200 Increase 10/300 GL size-exclusion chromatography (SEC) column at 6 °C. The running buffer was BRB80, supplemented with 1 mM GTP, at a flow rate of 0.5 mL/min. The 1 mL fraction with the highest eluted tubulin concentration (between 5 and 6 mg/mL) was collected and used for the experiments.

Buffer Exchange

We exchanged the buffer of the tubulin solution using a microcentrifuge column (Micro Bio-SpinTM P-6 Gel column, Bio-Rad) and a 5810 R Eppendorf centrifuge, at 4 °C. This SEC column adsorbs from the applied solution 99% (under our conditions) of the compounds whose molecular weight is 6 kD or less¹.

Each new column was reconditioned to BRB80 by adding 500 μ L BRB80, spinning down at 1000 g for 1 min. This process was repeated four times, followed by a final spin at 1000 g for 3 min to remove any excess buffer.

Between 20 and 50 μ L tubulin solution was then added to the column and spun at 1000 g for 4 min. The eluted tubulin solution maintained its initial volume whereas 99% of the free (unbound) nucleotides were removed². The buffer of the eluted tubulin solution was then

¹The small compounds are retained in the beads of the column, made of polyacrylamide gel (Bio-Gel P-6).

²At this stage, no more than 0.5% of the nucleotides dissociated from the tubulin dimer into the buffer (based on the values of K_1 - K_4 , see below).

exchanged again (using a reconditioned column), attaining an overall free nucleotide dilution factor of 10^4 .

Nucleotide Extraction by Protein Precipitation

Tubulin-bound nucleotides were extracted by denaturing and precipitating the protein, using a modified version of a published protocol.⁴⁸ Samples were placed on ice to quench GTP hydrolysis. Ice-cold 70% w/w aqueous perchloric acid (HClO_4) solution was then added at a final concentration of 0.5 M. A byproduct of the HClO_4 addition was salt sedimentation, also observed in tubulin-free control samples. When tubulin was present, the HClO_4 immediately denatured the protein³. For complete precipitation, the samples were incubated on ice for 10 min. The precipitants (denatured tubulin and salt) were then removed by centrifugation at 4 °C for 10 min at 14,000 g. The top 60% of the initial total solution volume was buffered by adding an ice-cold solution of 1 M K_2HPO_4 and 0.5 M acetic acid, whose volume was 10% of the initial solution volume. The perchloric acid was then neutralized by adding a similar volume of ice-cold 3 M KOH solution. These additions led to additional salt sedimentation. The samples were then stored at -20°C .

HPLC Analysis

Reverse-phase ion-pair high performance liquid chromatography (HPLC) analysis was performed at 25 °C using a Waters Alliance e2695 Separation Module (Waters Corporation, Milford, USA), equipped with a 2489 UV-Vis detector that recorded the absorbance at 260 nm. Following a published protocol,⁴⁸ the HPLC column was SupelcosilTM LC-18-DB, which was 25 cm long, had an internal diameter of 4.6 mm, and packed with 5 μm particles. The HPLC mobile phase contained 0.2 M K_2HPO_4 , 0.1 M acetic acid, and 4 mM tetrabutylammonium phosphate, at pH 6.7. At this pH, all the measured nucleotide species were charged (GTP,

³The HClO_4 led to tubulin aggregation on the tip of the pipette used for adding the acid and prevented mixing of the solution by pipetting up and down. In the absence of protein, we verified that the fact that we did not mix the solutions did not change the results.

GDP, guanosine monophosphate (GMP), and ATP had a negative charge of 4, 3, 2 and 4 e , respectively).⁴⁹

The mobile phase was filtered using Millex-GV syringe filter (Polyvinylidene fluoride (PVDF) membrane, 33 mm in diameter, and pore size of 0.22 μm). A flow-rate of 1 mL/min was used, unless otherwise is indicated. The column was equilibrated by flowing at least 40 mL of the filtered mobile phase.

Before HPLC analysis, frozen samples were thawed and centrifuged at 4 °C for 10 min at 20,800 g. The top 60% or less of the initial volume was diluted with the HPLC elution buffer to reach between 10 and 60 μM total nucleotide concentration. This range was selected to ensure good separation between the peaks at the HPLC elution profile, avoid saturation of the UV-Vis detector, and be sensitive to the nucleotide content in our lowest tubulin concentration (1 mg/mL).

A typical purified tubulin concentration of 36 mg/mL (0.31 mM, using a tubulin dimer molar mass of 116 kDa) had a bound nucleotide concentration of 0.62 ± 0.05 mM (assuming that two nucleotides were bound to each dimer). Addition of 0.5 M HClO_4 , 1 M K_2HPO_4 , 0.5 M acetic acid, and 3 M KOH diluted the nucleotide concentration to 0.45 mM. To be within the optimal HPLC working conditions, the sample was ten-fold diluted by the HPLC mobile phase to 45 μM , before it was injected into the HPLC.

Each HPLC experiment included calibration samples of mixed GTP, GDP, and GMP, dissolved in the HPLC mobile phase, filtered using PVDF membrane syringe filters, 13 mm in diameter and pore size of 0.2 μm (mda Membrane Technologies, Inc.). Figure 2 shows the elution profiles of a mixture of GTP, GDP, GMP, ATP, and guanosine (Gu). This mixture included the compounds of the calibration sample. The ATP elution peak appeared at 14.5 ± 0.1 mL (or 14.5 ± 0.1 min, as the flow rate was 1 mL/min) and was disregarded in our analyses (Figure 2). The column dead volume was 3.6 ± 0.1 mL (regardless of the injected sample).

When more than 9 samples were measured sequentially, the HPLC tubing and pumps

Figure 2: HPLC elution chromatograms of a nucleotide mixture containing $45 \pm 2 \mu\text{M}$ GMP, $40 \pm 2 \mu\text{M}$ GDP, $40 \pm 2 \mu\text{M}$ GTP, $40 \pm 2 \mu\text{M}$ Guanosine (Gu), and $35 \pm 2 \mu\text{M}$ ATP, prepared as explained in Purchased Nucleotides Purity subsection.

were wet-primed, using 40 mL water, followed by 20 mL of the HPLC mobile phase (using a flow rate of 4 mL/min). The column was then equilibrated by flowing 15 mL of the mobile phase at a flow rate of 1 mL/min. A calibration sample was then measured.

The HPLC peak area measurements were extremely precise, as can be seen by the error bars of Figure S1. Nevertheless, temperature variations of up to 5 °C between successive measurements could shift the position of the eluted peaks by 0.3 mL or less (Figure S3). An equilibration time of 25 min, could shift the position of the peaks by 5 mL or less (Figure S4). Prior to each set of measurements, the column was equilibrated with at least 40 mL of the filtered mobile phase at a flow rate of 1 mL/min (i.e an equilibration time of 40 min).

Nucleotide Content Measurement Protocols

GTP to GDP Molar Ratio

To determine the total (tubulin-bound and unbound) GTP to GDP molar ratio (in either tubulin or tubulin-free solutions), the Nucleotide Extraction by Protein Precipitation protocol was applied, followed by HPLC Analysis.

Simultaneous Measurements in the Buffer and on Tubulin

To isolate the tubulin-bound nucleotides from the free nucleotides, we applied the Buffer Exchange protocol into BRB80. The tubulin-bound GTP to GDP Molar Ratio was then determined and the GTP and GDP mole fractions at the E-site was calculated, assuming the nucleotide at the N-site was always GTP.

To determine the GTP to GDP molar ratio in the buffer, we collected the first 500 μ L of BRB80, from the first Buffer Exchange (out of two), applied to the tubulin sample. Based on UV-Vis measurements, about 60% of the free (unbound) nucleotides were eluted into the buffer. Depending on the volume of the loaded tubulin sample, the nucleotide concentration in the eluted buffer was 20- to 40-fold diluted. We then took an aliquot of 100 μ L (out of the 500 μ L) and determined its GTP to GDP Molar Ratio.

Protocol Validation and Control Measurements

Purchased Nucleotides Purity

The molar ratio between GTP, GDP, and GMP nucleotides was determined in the purchased GTP, GDP, and GMP powders (Figure S5). The powders were dissolved in the HPLC mobile phase, filtered through PVDF membrane syringe filters, and injected into the HPLC.

As was previously noted,^{39,48} impurity traces of GTP, GDP, and GMP, are shown in Figures S1 and S5, and summarized in Table 1.

Table 1: Nucleotide composition, in units of mole percent, of the purchased nucleotides, prepared as explained in Purchased Nucleotides Purity, determined by HPLC Analysis. The measurement precision was ± 1 mole percent.

	% GTP	% GDP	% GMP
GTP sample	96	4	0
GDP sample	1	95	4
GMP sample	0	0	100

Protocol Validation Measurements

Figure S1 shows that the GTP to GDP molar ratio of the purchased GTP, GDP, and GMP powders, determined by the protocol described in subsection GTP to GDP Molar Ratio, were comparable to those obtained by direct injection to the HPLC. Storage of the samples at -20°C or the initial nucleotide concentration did not change the GTP to GDP molar ratio (Figure S1).

Nucleotide Composition of Different Tubulin Preparation Protocols

The molar ratio of tubulin-bound GTP to tubulin-bound GDP (and the corresponding molar ratio at the tubulin E-site) was measured for several GTP- and GDP-tubulin preparation protocols (Table 2). The result after incubation with 10 mM GTP was similar to previously published data,⁵⁰ in which the GTP-tubulin complex was isolated by chromatography after

incubation in 0.5 mM GTP. The GTP in the earlier experiments,⁵⁰ however, was 99% pure, unlike our GTP that was only 96% pure (Table 1).

Seven heat-cool cycles had tubulin-bound GTP to GDP molar ratio similar to tubulin that was incubated in a BRB80 buffer, supplemented with 10 mM GDP. We attribute the residual (6%) tubulin-bound GTP in the sample, incubated in 10 mM GDP (Table 2), to the 0.5 ± 0.1 mM GTP in the purified tubulin sample, and to the GTP/GDP tubulin exchange reaction (Reaction 1).

Table 2: The composition of the tubulin-bound (and E-site-bound) nucleotides of purified 36 mg/mL tubulin samples, following different preparation protocols, explained in Sample Preparation Protocols. No GMP-tubulin fraction was detected in any of the samples. The measurement precision of the total bound nucleotide values was 1 mole percent (leading to a precision of 2 mole percent in the E-site values).

	% GTP-bound tubulin dimer (E-site)	% GDP-bound tubulin dimer (E-site)
Purified tubulin sample	80.5 (61)	19.5 (39)
Addition of 4 mM GTP	90 (80)	10 (20)
Addition of 10 mM GTP	92.5 (85)	7.5 (15)
Addition of 10 mM GDP	53 (6)	47 (94)
One cycle	65 (30)	35 (70)
Three cycles	59 (18)	41 (82)
Seven cycles	53 (6)	47 (94)

GDP was not added to the samples to which seven heat-cool cycles were applied. The tubulin-bound GTP (after the purification), was hydrolyzed in each cycle when the samples were incubated at 36 °C, during which spontaneous tubulin nucleation and MT assembly took place. The nucleation and assembly rates, however, decreased as the number of cycles proceeded because the GTP to GDP molar ratio of both tubulin-bound and tubulin-free nucleotides decreased (Table 2).²¹ As a result, during the last cycle or two, when the GTP to GDP molar ratio was low, the rates of nucleation and assembly were slower than the 35 min incubation period at 36 °C.

Results and Discussion

GTP Hydrolysis Kinetics

GTP Hydrolysis Was Negligible in the Absence of Tubulin

We examined the reaction of GTP hydrolysis

where P_i is the leaving phosphate (PO_4^{3-}) group and k_h is the hydrolysis rate constant. In the absence of tubulin, no hydrolysis was detected in the MT assembly buffer, unless the temperature was raised to 36 °C (Figure 3). At 36 °C, the hydrolysis reaction was fitted to a pseudo first-order kinetics with a k_h of $7.5 \pm 0.3 \times 10^{-8} \text{ s}^{-1}$. This implies that hydrolysis of free (or unbound) GTP was extremely slow and can be neglected in tubulin assembly kinetics experiments.

GTP Hydrolysis Was Not Involved in the Formation of Tubulin Oligomers and Single Rings

Tubulin solutions, eluted from SEC in an assembly buffer, did not hydrolyze GTP under conditions that did not allow spontaneous tubulin nucleation and MT assembly (Figure 4). Under these conditions, tubulin self-associated head-to-tail into 1D curved oligomers, terminated by the formation of tubulin single rings and the solutions were free of larger tubulin aggregates.^{12,14} Our data therefore suggest that GTP hydrolysis was not involved in the formation of 1D oligomers and rings.

In glycerol-supplemented assembly buffer, however, GTP hydrolysis was reported in tubulin solutions under conditions that did not allow spontaneous MT assembly. The hydrolysis was associated with the formation of tubulin oligomers.²¹

MT assembly rate, and consequently GTP hydrolysis rate, increase dramatically in the

Figure 3: GTP hydrolysis kinetics in the absence of tubulin. Solutions of 4 mM GTP in BRB80 were incubated at different temperatures, as indicated. The GTP to GDP molar ratio was measured as a function of time. The logarithm of the ratio between the GTP concentration at any time t ($[GTP]_t$) and the initial GTP concentration, ($[GTP]_0$), is plotted as a function of the incubation time (at time zero, the solution contained 0.2 mM GDP impurity, based on Table 1). Assuming a pseudo first-order hydrolysis kinetics at 36 °C (solid red diamonds), the slope of the broken red line corresponds to a rate constant, k_h , of $7.5 \pm 0.3 \times 10^{-8} \text{ s}^{-1}$. The horizontal broken black line is a guide for the eye, indicating the initial state of the solutions (Table S3).

presence of glycerol.⁵¹ This increase was previously attributed to the preferential hydration of tubulin in the presence of glycerol, which is excluded from the dimer. When glycerol is added, the standard chemical potentials of a free tubulin-dimer and a tubulin-dimer inside the MT lattice are both higher than the corresponding standard chemical potentials in the glycerol-free assembly buffer. In the presence of glycerol, however, the difference between the chemical potentials of the dimer in the free and the polymer states is larger. The greater stabilization of the MT polymer was attributed to the smaller excluded volume of glycerol in the assembled state.^{52,53} Glycerol does not alter the MT lattice structure.⁵⁴

It was also reported that tubulin aggregates are present in significant amounts in high-molarity glycerol assembly buffers. The GTP hydrolysis rate-constant in the presence of the aggregates is similar to that in the presence of MT.⁵¹ Hence it is likely that in the presence of glycerol under conditions that do not allow spontaneous MT assembly, tubulin aggregates, rather than tubulin oligomers, were involved in the hydrolysis of GTP.²¹

GTP Hydrolysis Above the Critical Conditions for MT Assembly

To examine the GTPase activity of tubulin, we analyzed GTP hydrolysis during MT nucleation and elongation in glycerol-free assembly buffer. GTP hydrolysis was detected at conditions that permitted spontaneous non-templated tubulin nucleation and MT assembly (Figure 4). In our SEC eluted tubulin concentrations, a pseudo first-order kinetics:⁵¹

$$\frac{[\text{GTP}]_t}{[\text{GTP}]_0} = \exp(-k_{\text{eff}}t), \quad (2)$$

with an effective GTP hydrolysis rate-constant of $k_{\text{eff}} = 1.27 \pm 0.02 \times 10^{-6} \text{ s}^{-1}$, adequately fitted our data (Figure 4). $[\text{GTP}]_t$ is the total GTP concentration (free and tubulin-bound) at time t and $[\text{GTP}]_0$ is the initial total GTP concentration. GTP hydrolysis occurs mainly on the MT lattice, hence k_{eff} was limited by the rate of MT assembly, determined during the nucleation phase.^{14,19}

In the presence of tubulin, the effective hydrolysis rate was about 20-fold faster than in the absence of tubulin (Figure 3). In the presence of both tubulin and glycerol, the hydrolysis was much faster ($4 \times 10^{-3} \text{ s}^{-1}$).¹⁹ In the absence of free nucleotides, nucleotide exchange reaction does not occur. Under those conditions, the GTP hydrolysis rates on a polymerized GTP-tubulin subunit, during the prenucleation phase (containing tubulin oligomers), interacting with a GTP-bound (homologous) or a GDP-bound tubulin (heterologous) neighbor were reported to be $3.8 \times 10^{-4} \text{ s}^{-1}$ and $1.3 \times 10^{-3} \text{ s}^{-1}$, respectively.²¹

The GTP hydrolysis rate is maximal during MT elongation (when the tubulin concentration is well above the critical tubulin concentration for MT elongation, $\sim 1 \text{ mg/mL}$). By adding preassembled MT seeds to a tubulin solution at different concentrations, the hydrolysis rate per MT was reported to be about 40 s^{-1} .³⁸ Under those conditions, the MT cap is about an order of magnitude larger than when the tubulin concentration is close to the critical concentration (where the cap is about ten dimers).^{38,55,56} Low Mg^{2+} concentrations and/or high GDP concentration decrease MT assembly and consequently the rate of GTP hydrolysis.^{25,57,58}

Tubulin-Bound Nucleotide Exchange Reaction

Apart from GTP hydrolysis, the tubulin-bound nucleotide composition can be varied by an exchange reaction between tubulin-bound and tubulin-free nucleotides. To investigate the exchange reaction, we used conditions at which GTP hydrolysis was negligible.

Tubulin samples were buffer exchanged to BRB80, containing 4 mM GTP, 2 mM GTP and 2 mM GDP, or 4 mM GDP, and incubated on ice for $\sim 2 \text{ h}$.

We then simultaneously measured the tubulin-bound GTP to GDP molar ratio and the unbound GDP to GTP molar ratio, $[\text{GDP}/\text{GTP}]_0$. The free nucleotides could be either bound to Mg^{2+} ions or Mg^{2+} -free. In a separate set of experiments, the tubulin solutions were eluted from SEC in BRB80, which was supplemented with 1 mM GTP. Nucleotides were then added to attain the final nucleotide composition. The measured mole fraction of

Figure 4: GTP hydrolysis kinetics in the presence of tubulin. Solutions of 5.2 ± 0.5 (open red squares) and 6.3 ± 0.3 mg/mL tubulin (open red circles and solid blue diamonds) were eluted from SEC column in glycerol-free BRB80, supplemented with 1 mM GTP. An aliquot of this solution was then diluted to 1.0 ± 0.1 mg/mL in the same buffer (solid blue triangles and open red hexagons). The GTP concentration was then increased to 4 ± 0.2 mM. The total GTP concentration (in the buffer and bound to the tubulin) was measured as a function of time at 4 °C (blue solid symbols) and 36 °C (red open symbols). $\ln \left(\frac{[\text{GTP}]_t}{[\text{GTP}]_0} \right)$ was plotted as a function of the incubation time and an effective pseudo first-order hydrolysis rate constant of $k_{\text{eff}} = 1.27 \pm 0.02 \times 10^{-6} \text{ s}^{-1}$ was fitted (broken line) to the data. The horizontal solid black line is a guide for the eye, indicating the initial state of each solution, before the temperature change (Table S4).

tubulin-bound GDP out of the total tubulin concentration, $\text{tub-GDP}_{\text{tot}}^{\%}$, was plotted against $[\text{GDP/GTP}]_0$ (Figures 5 and S7).

Figure 5: Mole fraction of GDP-tubulin out of the total tubulin concentration ($\text{tub-GDP}_{\text{tot}}^{\%}$, Equation S30), as a function of the molar ratio between tubulin-free GDP to tubulin-free GTP ($[\text{GDP/GTP}]_0$). The tubulin-free nucleotides may be either bound or unbound to Mg^{2+} ions. The reactions were performed at different added nucleotide compositions and total (ice-cold) tubulin concentrations. 1 (green), 6 (red), and 36 mg/mL (black open circle symbols) tubulin samples underwent Buffer Exchange for BRB80, supplemented with a total nucleotide concentration of 4 mM. In another set of experiments, ice cold tubulin solutions were eluted by SEC in BRB80, supplemented with 1 mM GTP, as described in SEC Protocol. The eluted fraction with the highest tubulin concentration was then diluted with the running buffer to 1 (green), 2 (blue), and 6 mg/mL (red solid squares), to which nucleotides were added to their final concentrations. The best fitted modeled mole fraction of GDP-tubulin (Equation S45) was computed based on Reactions 3-18, by solving Equation Sets S43 and S44, using the equilibrium constants which accounted for the difference between tubulin with exposed and buried E-sites (Table 3). The best fitted models are presented by curves in the colors of the corresponding data, where SEC elution and buffer exchanged models are presented as solid and broken curves, respectively. The measured HPLC data are provided in Tables S1 and S2.

Analyzing the Exchange Reaction Excluding the Contribution of Tubulin Self-Association

Initially, the data were fitted to the following set of coupled equilibrium reactions (Figure S7), suggested in an earlier study.⁴⁰

where 'tub' corresponds to tubulin whose E-site is nucleotide-free. tub-GTP and tub-GDP correspond to tubulin whose E-site is bound to GTP or GDP, respectively. As the nucleotides can complex with Mg^{2+} ions, the tub-GTP/GDP complexes could further bind a Mg^{2+} ion, leading to tub-GDP/GTP- Mg^{2+} complexes. We ignored the additional Mg^{2+} binding sites on the tubulin dimer (mainly at the carboxyl-terminal of both monomers⁴⁰), owing to their relatively weak binding constants ($\approx 100 \text{ M}^{-1}$).^{40,43} K_2 , K_5 , K_6 , and K_9 were taken from earlier measurements.^{40,59} K_7 and K_8 were computed from the other equilibrium constants:

$$\begin{aligned} K_7 &= \frac{K_3 K_5}{K_1}, \\ K_8 &= \frac{K_4 K_6}{K_2}. \end{aligned} \tag{12}$$

In Figure S7, the measured tub-GDP_{tot}[%] as a function of $[\text{GDP/GTP}]_0$, was fitted (using Equation S31) to reactions 3-11, excluding the contribution of tubulin self-association, assuming all of the tubulin dimers similarly reacted with the nucleotides and Mg^{2+} ions. The fit was obtained by solving Equation Sets S29 and S30, using the algorithm described in Excluding the Difference Between Tubulin Dimers With Exposed and Buried E-Sites section of the Supporting Information (SI). The best fitted equilibrium constants are provided in Table 3. The values of K_3 and K_4 are in agreement with those obtained in an earlier study, performed at 0 °C in 100 mM PIPES buffer (pH 6.9).⁴⁰ The fit to the data was weakly sensitive to the value of K_1 .

Analyzing the Exchange Reaction Including the Contribution of Tubulin Self-Association

In Figure S7, the fitted curves captured the general trend of the data set but missed the effect of tubulin concentration. At a fixed $[\text{GDP/GTP}]_0$, the measured tub-GDP_{tot}[%] increased with tubulin concentration, most likely, owing to the enhanced oligomerization of tubulin at higher concentrations.¹⁴ Below the critical MT assembly conditions, GTP- and GDP-tubulin assemble into small 1D assemblies.^{14,21} The observed increase in the mole fraction

of tubulin, buried in the small assemblies, with increasing total tubulin concentration and $[\text{GDP}/\text{GTP}]_0$, is consistent with stronger self-association energy of GDP-tubulin compared with GTP-tubulin.^{12,14,21}

In GDP-rich tubulin assemblies (1D curved oligomers and rings), the E-site is somewhat covered by the neighboring α -tubulin (green monomer and cyan GDP molecule in Figure 1) and the rate of nucleotide exchange is limited by the rate of oligomer dissociation.¹⁸ In the presence of MT-associated proteins (MAPs), tubulin dimers, incorporated into rings, were previously shown to prohibit nucleotide exchange reactions.²⁰ A similar block, most likely, occurred without MAPs and affected the degree of nucleotide exchange.¹⁸ Furthermore, simulations revealed that free GTP-tubulin dimers are more flexible than free GDP-tubulin dimers. Both dimers, however, were shown to assume a similar average curvature (or angle between monomers).⁶⁰ Besides, GTP-tubulin dimers were previously shown to retain their ability to exchange nucleotides while incorporated in a MT lattice.¹⁹

Taken together, these results suggest that tubulin oligomerization affects nucleotide exchange reaction. We, therefore, propose the following reasonable mechanism, illustrated in Figure 6. The E-site of the tubulin dimer can be either exposed to the solution or buried in the interdimer interface (Figure 1) within a tubulin assembly (an assembly of size n has $n - 1$ buried E-sites and one exposed E-site). We postulate that a nucleotide bound at an exposed E-site may be exchanged with different equilibrium constants than a nucleotide bound at a buried E-site. We should therefore include (in addition to Reactions 3-11, relevant for dimers with exposed E-site), the following set of exchange reactions (relevant for dimers with buried E-site):

Figure 6: Illustration of a possible mechanism of the nucleotide exchange reaction (Reaction 1). The mechanism includes 15 elementary steps (Reactions 3-11 and 13 - 18). The box shows the legends of the participating species. The center illustrates a tubulin solution. The cartoon was created using PyMOL.¹⁵

$$\text{tub}_{\text{Buried}} + \text{GTP-Mg}^{2+} \rightleftharpoons \text{tub}_{\text{Buried}}\text{-GTP-Mg}^{2+} \quad K_{12} = \frac{[\text{tub}_{\text{Buried}}\text{-GTP-Mg}^{2+}]}{[\text{tub}_{\text{Buried}}][\text{GTP-Mg}^{2+}]} \quad (15)$$

$$\text{tub}_{\text{Buried}} + \text{GDP-Mg}^{2+} \rightleftharpoons \text{tub}_{\text{Buried}}\text{-GDP-Mg}^{2+} \quad K_{13} = \frac{[\text{tub}_{\text{Buried}}\text{-GDP-Mg}^{2+}]}{[\text{tub}_{\text{Buried}}][\text{GDP-Mg}^{2+}]} \quad (16)$$

$$\text{tub}_{\text{Buried}}\text{-GTP} + \text{Mg}^{2+} \rightleftharpoons \text{tub}_{\text{Buried}}\text{-GTP-Mg}^{2+} \quad K_{14} = \frac{[\text{tub}_{\text{Buried}}\text{-GTP-Mg}^{2+}]}{[\text{tub}_{\text{Buried}}\text{-GTP}][\text{Mg}^{2+}]} \quad (17)$$

$$\text{tub}_{\text{Buried}}\text{-GDP} + \text{Mg}^{2+} \rightleftharpoons \text{tub}_{\text{Buried}}\text{-GDP-Mg}^{2+} \quad K_{15} = \frac{[\text{tub}_{\text{Buried}}\text{-GDP-Mg}^{2+}]}{[\text{tub}_{\text{Buried}}\text{-GDP}][\text{Mg}^{2+}]} \quad (18)$$

K_{14} and K_{15} were computed from the other equilibrium constants, according to the following equations:

$$\begin{aligned} K_{14} &= \frac{K_5 K_{12}}{K_{10}} \\ K_{15} &= \frac{K_6 K_{13}}{K_{11}} \end{aligned} \quad (19)$$

To reduce the number of free parameters, the ratio between the total tubulin concentration with exposed ($[\text{tub}_{\text{Exposed}}^{\text{tot}}]$) and buried E-sites ($[\text{tub}_{\text{Buried}}^{\text{tot}}]$) was determined by applying an isodesmic model of tubulin self-association into single rings and 1D curved oligomers, adopted from earlier publications (summarized in Thermodynamic Model of Tubulin Self-Association into Single Rings and Curved 1D Oligomers section in the SI).^{12,14,21,42,43} We have also assumed that the standard Helmholtz dimer-dimer association free energy per longitudinal contact, ΔF_c° , and for closing a curved oligomer into a ring, $\Delta F_{\text{RC}}^\circ$, vary with

$[\text{GDP/GTP}]_0$ (as shown in Figure S6 and explained in section Standard Helmholtz Association Free Energies in the SI). In addition, was assumed that 10% of the tubulin dimers formed stable rings when prepared according to the Buffer Exchange protocol.^{12,14}

In Figure 5, $\text{tub-GDP}_{\text{tot}}^{\%}$ is plotted as a function of $[\text{GDP/GTP}]_0$ and the data were fitted (using Equation S45) to reactions 3-11 and 13-18, by solving Equation sets S43 and S44, which take into account the difference between tubulin dimers with exposed and buried E-sites (Including the Difference Between Tubulin Dimers with Exposed and Buried E-sites section in the SI). The resulting equilibrium constants are presented in Table 3, based on which the mole fractions of all the species in each of the solutions were computed and plotted as a function of $[\text{GDP/GTP}]_0$ (Figures S8 - S20).

Figures 5, S8 and S11 show that when taking into account the assembly of tubulin, the model can explain the increase in $\text{tub-GDP}_{\text{tot}}^{\%}$ with increasing tubulin concentration. The thermodynamic model of tubulin self-association predicts that the mole fraction of buried tubulin dimers increases with increasing tubulin concentration (Figures S14 and S15). The effect is stronger in the Buffer Exchange protocol than in the SEC Protocol owing to the presence of stable tubulin single rings in the former protocol.¹⁴ Figure 7 illustrates the computed equilibrium state of a 6 mg/mL tubulin solution with $[\text{GDP/GTP}]_0 = 1$, where the model in Figure 5 fits the data well. The suggested reaction mechanism, however, misses the data at high $[\text{GDP/GTP}]_0$ values at low tubulin concentrations, indicating that the proposed mechanism is incomplete.

Mg^{2+} Ions Associated with Tubulin Primarily Via Their Initial Association With Free Nucleotides

The values of K_5 and K_6 indicate that in the absence of tubulin, Mg^{2+} ions bind more readily to GTP than to GDP. When 4 mM GTP are mixed with 1 mM Mg^{2+} , about 22% of the GTP complex with Mg^{2+} whereas when 4 mM GDP are mixed with 1 mM Mg^{2+} , the affinity to Mg^{2+} is weaker and only 17% of the GDP complex with Mg^{2+} (based on Reactions 7 and

Figure 7: Illustration of the computed equilibrium state of a 6 mg/mL tubulin solution with 2 mM GTP, 2 mM GDP, 1 mM EGTA, and 1 mM Mg^{2+} , where the model in Figure 5 fits the data well. The box shows the legends of the various participating species. Note that the proportion of GTP, GDP, Mg^{2+} , and EGTA molecules, with respect to tubulin subunits, is 100-fold smaller than their actual number in the solution. The cartoon was created using PyMOL.¹⁵

8).

Figure S16 shows that in the presence of tubulin and in excess GTP, between 80% and 90% of the Mg^{2+} ions bind tubulin-free GTP. Most of the remaining Mg^{2+} ions are free, and only at high tubulin concentration about 7% of the Mg^{2+} ions bind GTP-tubulin dimers whose E-site is buried in assemblies. In excess GDP, no more than 65% of the Mg^{2+} ions bind tubulin-free GDP and most of the remaining Mg^{2+} ions remain free. Only at high tubulin concentration, about 4% of the Mg^{2+} ions bind GDP-tubulin dimers whose E-site is buried in assemblies. When $[\text{GDP}/\text{GTP}]_0 = 1$, the mole fraction of $\text{GTP} \cdot \text{Mg}^{2+}$ is about 3 times larger than the mole fraction of $\text{GDP} \cdot \text{Mg}^{2+}$. In other words, the stronger affinity of Mg^{2+} to GTP is preserved also in the presence of tubulin.

Figure S11 shows that when excluding the contribution of tubulin self-association, the affinity of Mg^{2+} ions to GTP-tubulin is much stronger than its affinity to GDP-tubulin. When including the contribution of tubulin self-association, however, the Mg^{2+} affinity became weaker, yet still stronger to tub-GTP.

When including the contribution of tubulin self-association, we distinguished between tubulin dimers with buried and exposed E-sites. Figure S13 shows that under excess GTP, about 25% of the GTP-tubulin dimers with buried E-sites ($\text{Tub}_{\text{Buried}}\text{-GTP}$) bind Mg^{2+} ions. Under excess GDP, no more than 15% of the GDP-tubulin dimers with buried E-sites ($\text{Tub}_{\text{Buried}}\text{-GDP}$) bind Mg^{2+} ions. When $[\text{GDP}/\text{GTP}]_0 = 1$, the mole fraction of $\text{Tub}_{\text{Buried}}\text{-GTP} \cdot \text{Mg}^{2+}$ is about 3 times larger than the mole fraction of $\text{Tub}_{\text{Buried}}\text{-GDP} \cdot \text{Mg}^{2+}$. Figure S12 shows a similar trend for tubulin dimers with exposed E-sites. Taken together, these results show that the binding of Mg^{2+} ions to the tubulin-bound nucleotides is weakly affected by the presence of tubulin. In other words, the association of Mg^{2+} ions with tubulin is mainly a result of their association with the tubulin-free nucleotides.

Species Existing in Negligible Concentrations As previously noted,⁴⁰ the chelation reaction of EGTA with Mg^{2+} can be neglected. Using the published equilibrium constant

($K_9 = 3.2 \text{ M}^{-1}$),⁵⁹ in the absence of tubulin and nucleotides, if 1 mM EGTA is mixed with 1 mM Mg^{2+} (as in our buffer), the EGTA will chelate 0.3 % of Mg^{2+} ions. Figure S16 shows that the binding of EGTA to Mg^{2+} remains negligible also in the presence of tubulin and nucleotides. In addition, Figures S8 - S14 show that under the conditions of our experiments the mole fraction of nucleotide-free tubulin dimers (tub) is negligible.

Table 3: The equilibrium constants that best fitted the HPLC data. K_2, K_5, K_6 , and K_9 were adopted from.^{39,40,59} K_7 and K_8 were calculated according to Equations 12. K_{14} and K_{15} were calculated according to Equations 19.

Equilibrium constant	Excluding the difference between tubulin dimers with exposed and buried E-sites	Including the difference between tubulin dimers with exposed and buried E-sites
K_1	$1.8 \pm 0.5 \times 10^7 \text{ M}^{-1}$	$1 \pm 0.5 \times 10^8 \text{ M}^{-1}$
K_2	$1.6 \times 10^7 \text{ M}^{-1}$	$1.6 \times 10^7 \text{ M}^{-1}$
K_3	$6.4 \pm 1 \times 10^7 \text{ M}^{-1}$	$6.8 \pm 5 \times 10^7 \text{ M}^{-1}$
K_4	$2.5 \pm 0.5 \times 10^7 \text{ M}^{-1}$	$1.9 \pm 1 \times 10^7 \text{ M}^{-1}$
K_5	2830 M^{-1}	2830 M^{-1}
K_6	675 M^{-1}	675 M^{-1}
K_7	$1 \pm 0.5 \times 10^4 \text{ M}^{-1}$	$1.9 \pm 1 \times 10^3 \text{ M}^{-1}$
K_8	$1.1 \pm 0.5 \times 10^3 \text{ M}^{-1}$	$8 \pm 3 \times 10^2 \text{ M}^{-1}$
K_9	3.2 M^{-1}	3.2 M^{-1}
K_{10}	-	$7 \pm 5 \times 10^7 \text{ M}^{-1}$
K_{11}	-	$1.4 \pm 0.5 \times 10^8 \text{ M}^{-1}$
K_{12}	-	$8.8 \pm 5 \times 10^7 \text{ M}^{-1}$
K_{13}	-	$1 \pm 0.5 \times 10^8 \text{ M}^{-1}$
K_{14}	-	$3.6 \pm 2 \times 10^3 \text{ M}^{-1}$
K_{15}	-	$4.8 \pm 2 \times 10^2 \text{ M}^{-1}$

Conclusions

We showed that GTP hydrolysis was negligible in the absence of tubulin and was not involved in the formation of tubulin 1D oligomers and single rings. Above the critical conditions for microtubule assembly, in glycerol-free assembly buffer, GTP hydrolysis followed a pseudo first-order kinetics with an effective rate constant of $1.27 \pm 0.02 \times 10^{-6} \text{ s}^{-1}$, limited by the rate of tubulin nucleation and assembly. This effective hydrolysis rate is much slower than the rate of GTP hydrolysis in the presence of glycerol, microtubule seeds, or in the absence of free nucleotides. Below the critical conditions for microtubule assembly, ice-cold tubulin dimers self-associated and a fraction of their nucleotide exchangeable sites (E-site) was buried. GTP to GDP exchange reaction occurred to a different extent on tubulin dimers with buried E-sites than on dimers with exposed E-sites. Our analysis showed that the association of Mg^{2+} ions with tubulin was primarily achieved via their association with the tubulin-free nucleotides. Understanding GTP hydrolysis and nucleotide-exchange reactions is critical for analyzing tubulin self-association, nucleation, and microtubule assembly dynamics.

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Graphical TOC Entry

Supporting Information

Effect of Tubulin Self-Association on GTP Hydrolysis and Nucleotide Exchange Reactions

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Running header

GTP Hydrolysis and Nucleotide Exchange

Nucleotide Composition Variation of Different Sample Preparation Protocols

The protein sedimentation protocol, its storage at -20°C following sedimentation, and the neutralization of the sample did not affect the measured mole fractions of GTP, GDP, and GMP. Figure S1 shows that the differences that were measured are marginal and within the experimental error.

Figure S1: Mole percent of GTP, GDP, and GMP of the nucleotide powders, dissolved in BRB80 buffer. Fresh (unlike frozen) samples were processed without the -20°C storage stage in Nucleotide Extraction by Protein Precipitation protocol (see Materials and Methods). Low ($30\text{ }\mu\text{M}$) and high conc. (0.8 mM) notations refer to the initial GTP and GDP concentrations. High concentration samples were 50-fold diluted before measurement, resulting in $\approx 15\text{ }\mu\text{M}$ nucleotide concentration in the samples that were injected into the HPLC. Low concentration samples were injected into the HPLC without further dilution. The errors and average values of each column were approximated from triplet experiments. No extraction refers to samples that were only dissolved in the HPLC mobile phase and filtered (using PVDF membrane syringe filters).

Variation in the HPLC Elution Profiles

Figure S2 shows that the locations of the HPLC elution peaks were weakly sensitive to repeated injections and did not lead to overlap between the GDP and GTP peaks. The variations in the area under the peaks are shown in Figure S1.

Variations in the elution profile owing to changes in the temperature and equilibration time were also measured (Figures S3 and S4). Whereas a temperature change of 5 °C did not lead to peak overlap, short equilibration periods resulted in a poor separation between the GTP and GDP peaks and decreased the precision of their molar ratio measurements.

When no peak overlap was detected, peak assignment was done according to the calibration sample (see HPLC Analysis in the Materials and Methods section), measured every 2 hours. Therefore, the shifts in the position of the peaks did not affect the analysis.

Figure S2: HPLC chromatograms of repeated injections of GTP and GDP solutions. The figure shows the shifts in the center of the main elution peak of six repeated injections of 15 μ M (A) and 30 μ M (B) GTP solutions, and six repeated injections of 15 μ M (C) and 30 μ M (D) GDP solutions. (E) The elution profiles of the 15 μ M GTP solution from A with their small GDP peak. The GDP and GTP peaks were separated by an elution volume of ≈ 1 mL, whereas the shifts in the locations of the peaks were significantly smaller.

Figure S3: Variations in the nucleotide elution profiles owing to temperature change.

Figure S4: Variations in the nucleotide elution profiles owing to different HPLC column equilibration times. A mixed solution of GTP, GDP, GMP, Guanosine and ATP (as in Figure 2) was injected into the HPLC column, following 25 or 55 min equilibration time at a flow rate of 1 mL/min. The column was kept in water before equilibration in the mobile phase. Short equilibration periods resulted in poor separation and overlap between guanosine and ATP elution peaks. Long equilibration times were required owing to the large dimensions of the column (4.2 mL inner volume) and the ion pair reagent that was used, tetrabutylammonium phosphate, a quaternary-ammonium salt with relatively long alkyl groups.¹

Figure S5: HPLC elution chromatograms of 24 μ M GTP (red), 24 μ M GDP (green), and 30 μ M GMP (black), dissolved in the HPLC mobile phase (see HPLC Analysis) and filtered (using PVDF membrane syringe filters). Trace amount of GDP was detected in the GTP powder, and trace amounts of GTP and GMP were present in the GDP powder. Table 1 summarizes the nucleotide compositions in each of the samples.

Thermodynamic Model of Tubulin Self-Association into Single Rings and Curved 1D Oligomers

Our model assumes equilibrium between free dimers, curved oligomers, and tubulin single rings, described by the following set of coupled assembly reactions:

n is the number of tubulin dimers, D , assemble into D_n curved tubulin oligomers (with $n - 1$ dimer - dimer contacts), and R_N is a tubulin single ring, containing $N = 13$ dimers (with N dimer-dimer contacts). ν_n , ν_R , and ν_D are the stoichiometric coefficients of D_n , R_N , and D respectively.

We aim to determine the distribution of tubulin assemblies at equilibrium. At temperature T , the equilibrium probability distribution of D_n and R_N structures in the canonical ensemble (NVT) depends on the standard Helmholtz dimer-dimer association free energy per longitudinal contact, ΔF_c° , and the standard Helmholtz free energy cost for closing a curved oligomer of length N into a ring, beyond the energy gain from the formation of an additional longitudinal dimer-dimer contract, ΔF_{RC}° . To obtain the distribution in the grand canonical ensemble (μVT), the translational entropy of the assembled structures has to be taken into account.

According to earlier theoretical models,²⁻⁶ we assumed an implicit solvent background (instead of explicitly considering the contribution of the water molecules or ions to the free energy). The tubulin dimer molecules could be in each of the assembly states. The concentration of each assembly was described on the mole fraction scale, where X_n , is the mole fraction of tubulin dimers, incorporated into a curved tubulin oligomer, containing n dimers, and is given by $X_n = nN_n/N_{\text{Total}}$. N_n is the number of 1D tubulin oligomers of size n , and N_{Total} is the total number of molecules. X_R is the mole fraction of tubulin dimers in

rings, given by $X_R = N \times N_R / N_{\text{Total}}$, where N_R is the number of single tubulin rings (containing N dimers). $N_{\text{Total}} = N_{\text{Tubulin}} + N_{\text{water}}$, where N_{Tubulin} is the total number of tubulin dimers in all of the tubulin assemblies and N_{water} is the total number of water molecules.

From the conservation of mass, we get,

$$X_R + \sum_{n=1}^N X_n = X_{\text{Total}}^{\text{Tubulin}} \quad (\text{S1})$$

where $X_{\text{Total}}^{\text{Tubulin}}$ is the total mole fraction of tubulin dimers.

We have shown⁷ that the mole fractions that minimize the total Helmholtz free energy in the grand canonical ensemble are

$$\begin{aligned} X_n &= n X_1^n \exp\left(-\frac{(n-1)\Delta F_c^\circ}{k_B T}\right) \\ X_R &= N X_1^N \exp\left(-\frac{\Delta F_{\text{RC}}^\circ + N\Delta F_c^\circ}{k_B T}\right) \end{aligned} \quad (\text{S2})$$

where k_B is Boltzmann's constant, and T is the absolute temperature.

From the conservation of mass we get

$$\begin{aligned} &N X_1^N \exp\left(-\frac{\Delta F_{\text{RC}}^\circ + N\Delta F_c^\circ}{k_B T}\right) + \\ &\sum_{n=1}^N n X_1^n \exp\left(-\frac{(n-1)\Delta F_c^\circ}{k_B T}\right) = X_{\text{Total}}^{\text{Tubulin}}. \end{aligned} \quad (\text{S3})$$

Solving Equation S3 for X_1 , using the experimental value of $C_{\text{Total}}^{\text{Tubulin}}$ and assuming different values of $\Delta F_{\text{RC}}^\circ$ and ΔF_c° , result in the entire mole fraction distribution.

The total mole fraction of tubulin dimers in solution is defined as:⁸

$$X_{\text{Total}}^{\text{Tubulin}} = \frac{C_{\text{Total}}^{\text{Tubulin}} N_{\text{AV}}}{M_W^{\text{Tubulin}} \times N_{\text{Total}}}, \quad (\text{S4})$$

where $C_{\text{Total}}^{\text{Tubulin}}$ is the total tubulin concentration in units of mg/mL (or g/L), and N_{AV} is the Avogadro number. Using standard protein specific volume of $v_{\text{protein}} = 0.75 \text{ mL/g}$,⁹ and the

molecular mass of a tubulin dimer, $M_W^{\text{Tubulin}} = 116000 \text{ g/mol}$, we get:

$$\begin{aligned}
V_{\text{Tubulin}} &= v_{\text{protein}} C_{\text{Total}}^{\text{Tubulin}} V_{\text{Total}} \\
N_{\text{Total}} &= N_{\text{Tubulin}} + N_{\text{Water}} = \\
&\frac{N_{\text{AV}} C_{\text{Total}}^{\text{Tubulin}} V_{\text{Total}}}{M_W^{\text{Tubulin}}} + \\
&\frac{N_{\text{AV}} (V_{\text{Total}} - V_{\text{Tubulin}}) \rho_{\text{Water}}}{M_W^{\text{Water}}}.
\end{aligned} \tag{S5}$$

where V_{Total} is the total solution volume.

To account for tubulin rings that did not disassemble owing to slow kinetics, the total mole fraction of tubulin dimers, $X_{\text{Total}}^{\text{Tubulin}}$, was modified to:

$$X_{\text{Total}}^{\text{Tubulin}} = \frac{C_{\text{Total}}^{\text{Tubulin}} (1 - X_{\text{Stable Rings}}) N_{\text{AV}}}{M_W^{\text{Tubulin}} \times N_{\text{Total}}}, \tag{S6}$$

where $X_{\text{Stable Rings}}$ is the mole fraction of stable rings out of the total tubulin concentration:

$$X_{\text{Stable Rings}} = \frac{C_{\text{Stable Rings}}}{C_{\text{Total}}^{\text{Tubulin}}}, \tag{S7}$$

for which $C_{\text{Stable Rings}}$ is the concentration of stable rings in units of mg/mL.

The concentration of buried tubulin dimers was calculated by:

$$\begin{aligned}
[\text{tub}_{\text{Buried}}^{\text{tot}}] &= C_{\text{Total}}^{\text{Tubulin}} (1 - X_{\text{Stable Rings}}) \times \\
&\frac{\sum_{n=1}^N \left(\frac{n-1}{n} X_n \right) + X_{\text{R}}}{X_{\text{Total}}^{\text{Tubulin}}} + C_{\text{Stable Rings}},
\end{aligned} \tag{S8}$$

where the concentration of tubulin with exposed E-sites is simply

$$[\text{tub}_{\text{Exposed}}^{\text{tot}}] = C_{\text{Total}}^{\text{Tubulin}} - [\text{tub}_{\text{Buried}}^{\text{tot}}]. \tag{S9}$$

Standard Helmholtz Association Free Energies

The standard Helmholtz longitudinal dimer-dimer association free energy per contact, ΔF_c° , is a function of the molar ratio between tubulin-free GDP to tubulin-free GTP, $[\text{GDP}/\text{GTP}]_0$. The tubulin-free nucleotides may still bind a Mg^{2+} ion. For $[\text{GDP}/\text{GTP}]_0 < 0.5$, ΔF_c° was 13.1 k_BT and 13.7 k_BT after applying the SEC Protocol and the Buffer Exchange protocol, respectively (see Materials and Methods). The standard Helmholtz free energy cost for closing a curved oligomer into a ring was $\Delta F_{\text{RC}}^\circ = -6.9$ k_BT. For $[\text{GDP}/\text{GTP}]_0 > 5$, $\Delta F_c^\circ = 14.6$ k_BT and $\Delta F_{\text{RC}}^\circ = -9.2$ k_BT were used. These energies were adopted from our earlier work.⁷

For $0.5 < [\text{GDP}/\text{GTP}]_0 < 5$, ΔF_c° was approximated to be 13.8 k_BT and 14.2 k_BT after applying the SEC Protocol and the Buffer Exchange protocol, respectively. $\Delta F_{\text{RC}}^\circ$ was estimated to be -8.1 k_BT. These energies were selected to best describe the experimental data. The free energies profiles were then smoothed using the tanh function,¹⁰ as explained in the following section.

Smoothing the Free Energy Profiles

To smooth the stepwise change in the standard Helmholtz free energies as a function of the molar ratio between tubulin-free GDP to tubulin-free GTP ($[\text{GDP}/\text{GTP}]_0$) (described in the earlier section), the following tanh smoothing¹⁰ function was used

$$\begin{aligned} \Delta F_c^\circ ([\text{GDP}/\text{GTP}]_0) = & \Delta F_c^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}}) + \\ & \frac{\Delta \Delta F_c^\circ}{2} \left(\tanh \left(\alpha \left(\log_{10} ([\text{GDP}/\text{GTP}]_0) - \right. \right. \right. \\ & \left. \left. \left. \log_{10} ([\text{GDP}/\text{GTP}]_{0,\text{mid}}) \right) \right) + 1 \right), \end{aligned} \quad (\text{S10})$$

where

$$\begin{aligned} \Delta \Delta F_c^\circ \equiv & \Delta F_c^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GDP}}) - \\ & \Delta F_c^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}}), \end{aligned} \quad (\text{S11})$$

and $\Delta F_c^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}})$ was 13.1 k_BT and 13.7 k_BT for the SEC Protocol and Buffer Exchange protocol, respectively (see Materials and Methods). $\Delta F_c^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GDP}})$ was 14.6 k_BT for both preparations. $[\text{GDP}/\text{GTP}]_{0,\text{GTP}}$ refers to $[\text{GDP}/\text{GTP}]_0 \rightarrow 0$, and $[\text{GDP}/\text{GTP}]_{0,\text{GDP}}$ to $[\text{GDP}/\text{GTP}]_0 \rightarrow \infty$. α was kept constant as 1/0.35, to ensure the smoothing was confined to $0.1 < [\text{GDP}/\text{GTP}]_0 < 10$. $[\text{GDP}/\text{GTP}]_{0,\text{mid}}$ was calculated so that $\Delta F_c^\circ ([\text{GDP}/\text{GTP}]_0 = 1.1)$ was 13.8 k_BT or 14.2 k_BT for the SEC Protocol or Buffer Exchange protocol, respectively.

$\log_{10}$ was applied on the $[\text{GDP}/\text{GTP}]_0$ to maintain the tanh function symmetric with respect to the most populated $[\text{GDP}/\text{GTP}]_0 = 1.1$ middle point, on a semilogarithmic plot (Figure S6).

The standard Helmholtz free energy cost for closing a curved oligomer into a ring was calculated using a similar approximation:

$$\begin{aligned} \Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_0) = & \\ \Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}}) + & \\ \frac{\Delta \Delta F_{\text{RC}}^\circ}{2} \left(\tanh \left(\alpha \left(\log_{10} ([\text{GDP}/\text{GTP}]_0) - \right. \right. \right. & \quad (S12) \\ \left. \left. \left. \log_{10} ([\text{GDP}/\text{GTP}]_{0,\text{mid}}) \right) \right) + 1 \right), & \end{aligned}$$

where

$$\begin{aligned} \Delta \Delta F_{\text{RC}}^\circ = \Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GDP}}) - & \\ \Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}}), & \quad (S13) \end{aligned}$$

and $\Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GTP}})$ was -6.9 k_BT and $\Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_{0,\text{GDP}})$ was -9.2 k_BT.

As for $\Delta F_c^\circ ([\text{GDP}/\text{GTP}]_0)$, α was kept constant as 1/0.35, while $[\text{GDP}/\text{GTP}]_{0,\text{mid}}$ was calculated so $\Delta F_{\text{RC}}^\circ ([\text{GDP}/\text{GTP}]_0 = 1.1)$ was -8.1 k_BT.

Figure S6 shows the variation in the standard Helmholtz free energies as a function of $[\text{GDP}/\text{GTP}]_0$.

Figure S6: (A) The standard Helmholtz dimer-dimer association free energy per longitudinal contact, ΔF_c° , and (B) the standard Helmholtz free energy cost for closing a curved oligomer into a ring, ΔF_{RC}° , as a function of $[GDP/GTP]_0$.

Solving the Set of Equilibrium Reactions

Excluding the Difference Between Tubulin Dimers With Exposed and Buried E-Sites

GTP and GDP can interact with Mg^{2+} ions. Hence, the affinity of tubulin towards GTP and GDP was previously suggested to include the following set of equilibrium reactions:¹¹

where tub corresponds to tubulin dimer whose E-site is nucleotide-free. tub-GTP/GDP correspond to tubulin dimer whose E-site is bound to GTP/GDP. As the nucleotides can complex with Mg^{2+} ions, the tub-GTP/GDP complexes can further bind a Mg^{2+} ion, leading to tub-GDP/GTP- Mg^{2+} complexes. K_7 and K_8 can be calculated by:

$$\begin{aligned} K_7 &= \frac{K_3 K_5}{K_1} \\ K_8 &= \frac{K_4 K_6}{K_2} \end{aligned} \quad (\text{S23})$$

Besides, the following mass conservation equations apply.

$$\begin{aligned} [\text{tub}_{\text{tot}}] &= [\text{tub}] + [\text{tub-GTP}] + \\ &[\text{tub-GDP}] + [\text{tub-GTP-Mg}^{2+}] + \\ &[\text{tub-GDP-Mg}^{2+}] \end{aligned} \quad (\text{S24})$$

$$\begin{aligned} [\text{Mg}_{\text{tot}}^{2+}] &= [\text{Mg}^{2+}] + [\text{GTP-Mg}^{2+}] + \\ &[\text{GDP-Mg}^{2+}] + [\text{tub-GTP-Mg}^{2+}] + \\ &[\text{tub-GDP-Mg}^{2+}] + [\text{EGTA-Mg}^{2+}] \end{aligned} \quad (\text{S25})$$

$$[\text{GTP}_{\text{tot}}^{\text{free}}] = [\text{GTP}] + [\text{GTP-Mg}^{2+}] \quad (\text{S26})$$

$$[\text{GDP}_{\text{tot}}^{\text{free}}] = [\text{GDP}] + [\text{GDP-Mg}^{2+}] \quad (\text{S27})$$

$$[\text{EGTA}_{\text{tot}}] = [\text{EGTA}] + [\text{EGTA-Mg}^{2+}] \quad (\text{S28})$$

After some algebra, we get equations that only depend on the concentration of unbound

Mg²⁺ ions:

$$[\text{GTP}] = \frac{[\text{GTP}_{\text{tot}}^{\text{free}}]}{1 + K_5 [\text{Mg}^{2+}]}$$

$$[\text{GDP}] = \frac{[\text{GDP}_{\text{tot}}^{\text{free}}]}{1 + K_6 [\text{Mg}^{2+}]}$$

$$[\text{EGTA}] = \frac{[\text{EGTA}_{\text{tot}}]}{1 + K_9 [\text{Mg}^{2+}]} \quad (\text{S29})$$

$$[\text{tub}] = \frac{[\text{tub}_{\text{tot}}]}{1 + K_1 [\text{GTP}] + K_2 [\text{GDP}] + K_3 K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_4 K_6 [\text{Mg}^{2+}] [\text{GDP}]}$$

$$[\text{Mg}_{\text{tot}}^{2+}] = [\text{Mg}^{2+}] (1 + K_5 [\text{GTP}] + K_6 [\text{GDP}] + K_3 K_5 [\text{tub}] [\text{GTP}] + K_4 K_6 [\text{tub}] [\text{GDP}] + K_9 [\text{EGTA}]).$$

We numerically solved Equation Set S29 for $[\text{Mg}^{2+}]$, for each experimental condition defined by $[\text{GTP}_{\text{tot}}^{\text{free}}]$, $[\text{GDP}_{\text{tot}}^{\text{free}}]$, $[\text{EGTA}_{\text{tot}}]$, $[\text{Mg}_{\text{tot}}^{2+}]$, and $[\text{tub}_{\text{tot}}]$. The numerical solution was calculated using *fzero* function of the Matlab[®] software. Using the calculated $[\text{Mg}^{2+}]$ we were able to calculate the GDP-tubulin mole fraction out of the total tubulin content, by

the following equation set:

$$\begin{aligned}
[\text{GTP}] &= \frac{[\text{GTP}_{\text{tot}}^{\text{free}}]}{1 + K_5 [\text{Mg}^{2+}]} \\
[\text{GDP}] &= \frac{[\text{GDP}_{\text{tot}}^{\text{free}}]}{1 + K_6 [\text{Mg}^{2+}]} \\
[\text{tub}] &= \frac{[\text{tub}_{\text{tot}}]}{1 + K_1 [\text{GTP}] + K_2 [\text{GDP}] + K_3 K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_4 K_6 [\text{Mg}^{2+}] [\text{GDP}]} \quad (\text{S30}) \\
\text{tub-GDP}_{\text{tot}}^{\%} &= \frac{[\text{tub-GDP}] + [\text{tub-GDP-Mg}^{2+}]}{[\text{tub}_{\text{tot}}]} = \\
&\quad \frac{K_2 [\text{tub}] [\text{GDP}] + K_4 K_6 [\text{tub}] [\text{GDP}] [\text{Mg}^{2+}]}{[\text{tub}_{\text{tot}}]}
\end{aligned}$$

The GDP-tubulin mole fraction ($\text{tub-GDP}_{\text{tot}}^{\%}$) was fitted to the experimental data by varying K_1 , K_3 and K_4 , to minimize Equation S31. The best fitted equilibrium constants are presented in Table 3 and the best fitted GDP-tubulin mole fraction curve is shown in Figure S7. K_2 , K_5 , K_6 , and K_9 were adopted from previous works.^{11,12} The minimization was done by the *fmincon* function of the Matlab[®] software, using the following cost function

$$\begin{aligned}
\text{Residuals} &= \\
\frac{1}{L} \sum_{l=1}^L &\left(\frac{\text{tub-GDP}_{\text{tot,Measured}}^{\%} \left([\text{GDP/GTP}]_{0,l} \right) - \text{tub-GDP}_{\text{tot,Modeled}}^{\%} \left([\text{GDP/GTP}]_{0,l} \right)}{\sigma_l} \right)^2 \quad (\text{S31})
\end{aligned}$$

where L is the number of experimental data points, and $\text{tub-GDP}_{\text{tot}}^{\%}$ is the mole fraction of GDP-tubulin (including tubulin that is both bound and unbound to Mg^{2+}) out of the total

tubulin content. σ_i is the experimental error of each data point.

In Figure S7, the mole fraction of GDP-tubulin as a function of $[\text{GDP/GTP}]_0$ data was fitted (using Equation S31) to reactions 3-11, excluding the difference between tubulin dimers with exposed and buried E-sites, by solving Equation Sets S29 and S30, as was done in an earlier publication.¹¹ In Figure 5 the same data were fitted (using Equation S45) to reactions 3-11 and 13-18, by solving Equation Sets S43 and S44, including the difference between tubulin dimers with exposed and buried E-sites.

Figure S7: The mole fraction of GDP-tubulin (out of the total tubulin concentration), as a function of $[GDP/GTP]_0$. The reactions were performed at different added nucleotide compositions and total (ice-cold) tubulin concentrations. 1 (green), 6 (red), and 36 mg/mL (black open circle symbols) tubulin samples were buffer exchanged for BRB80, supplemented with a total nucleotide concentration of 4 mM (as explained in Buffer Exchange in the Materials and Methods section). In another set of experiments, ice cold tubulin solutions were eluted from SEC in BRB80, supplemented with 1 mM GTP, as described in SEC Protocol in the Materials and Methods section. The eluted fraction with the highest tubulin concentration was then diluted with the running buffer to reach 1 (green), 2 (blue), and 6 mg/mL (red solid square symbols), to which we added either 3 mM GTP, or 1 mM GTP and 2 mM GDP. The entire data set was simultaneously fitted to Reactions 3-11, by solving Equation Sets S29 and S30, excluding the difference between tubulin dimers with buried and exposed E-sites. The best-fitted equilibrium constants are shown in Table 3. The best fitted curves are presented with the same color as the corresponding solid symbol data points. The measured HPLC data are provided in Tables S1 and S2.

Including the Difference Between Tubulin Dimers with Exposed and Buried E-sites

To improve the fit to the data in Figure S7 (that was based on reactions 3-11¹¹), we distinguished between tubulin dimers exposed ($\text{tub}_{\text{Exposed}}$) and buried ($\text{tub}_{\text{Buried}}$) E-sites in 1D curved assemblies (Figure 1). The total mole fraction of dimers with exposed E-site was calculated according to Equation S9 in section Thermodynamic Model of Tubulin Self-Association into Single Rings and Curved 1D Oligomers. Dimers with exposed E-site followed Reactions S14-S22 whereas in parallel, dimers with buried E-sites followed an analogous set of reactions:

As a result, the tubulin mass conservation equations are

$$[\text{tub}_{\text{tot}}] = [\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}], \quad (\text{S36})$$

where

$$\begin{aligned}
[\text{tub}_{\text{Exposed}}^{\text{tot}}] &= [\text{tub}_{\text{Exposed}}] + [\text{tub}_{\text{Exposed}}-\text{GTP}] + \\
&[\text{tub}_{\text{Exposed}}-\text{GDP}] + [\text{tub}_{\text{Exposed}}-\text{GTP}-\text{Mg}^{2+}] + \\
&[\text{tub}_{\text{Exposed}}-\text{GDP}-\text{Mg}^{2+}]
\end{aligned} \tag{S37}$$

and

$$\begin{aligned}
[\text{tub}_{\text{Buried}}^{\text{tot}}] &= [\text{tub}_{\text{Buried}}] + [\text{tub}_{\text{Buried}}-\text{GTP}] + \\
&[\text{tub}_{\text{Buried}}-\text{GDP}] + [\text{tub}_{\text{Buried}}-\text{GTP}-\text{Mg}^{2+}] + \\
&[\text{tub}_{\text{Buried}}-\text{GDP}-\text{Mg}^{2+}]
\end{aligned} \tag{S38}$$

In addition, the $\text{Mg}_{\text{tot}}^{2+}$, $\text{GTP}_{\text{tot}}^{\text{free}}$, $\text{GDP}_{\text{tot}}^{\text{free}}$, and EGTA_{tot} mass conservation equations are

$$\begin{aligned}
[\text{Mg}_{\text{tot}}^{2+}] &= [\text{Mg}^{2+}] + [\text{GTP}-\text{Mg}^{2+}] + \\
&[\text{GDP}-\text{Mg}^{2+}] + [\text{tub}_{\text{Exposed}}-\text{GTP}-\text{Mg}^{2+}] + \\
&[\text{tub}_{\text{Exposed}}-\text{GDP}-\text{Mg}^{2+}] + \\
&[\text{tub}_{\text{Buried}}-\text{GTP}-\text{Mg}^{2+}] + \\
&[\text{tub}_{\text{Buried}}-\text{GDP}-\text{Mg}^{2+}] + [\text{EGTA}-\text{Mg}^{2+}]
\end{aligned} \tag{S39}$$

$$[\text{GTP}_{\text{tot}}^{\text{free}}] = [\text{GTP}] + [\text{GTP}-\text{Mg}^{2+}] \tag{S40}$$

$$[\text{GDP}_{\text{tot}}^{\text{free}}] = [\text{GDP}] + [\text{GDP}-\text{Mg}^{2+}] \tag{S41}$$

$$[\text{EGTA}_{\text{tot}}] = [\text{EGTA}] + [\text{EGTA}-\text{Mg}^{2+}] \tag{S42}$$

where $[\text{tub}_{\text{Exposed}}^{\text{tot}}]$ is the total tubulin concentration with exposed E-sites, and $[\text{tub}_{\text{Buried}}^{\text{tot}}]$ is the total tubulin concentration with buried E-sites. The ratio between $[\text{tub}_{\text{Exposed}}^{\text{tot}}]$ and

$[\text{tub}_{\text{Buried}}^{\text{tot}}]$ is determined by the 1D curved oligomerization thermodynamic model of tubulin self-association⁷ (see Section Thermodynamic Model of Tubulin Self-Association into Single Rings and Curved 1D Oligomers and Figure S15), where in a 1D assembly of size n there are $n - 1$ tubulin dimers with buried E-sites and a dimer with an exposed E-site. In a closed tubulin single ring, all the dimers have buried E-sites.

After some algebra, the resulting set of equations, which only depend on $[\text{Mg}^{2+}]$, is

$$\begin{aligned}
[\text{GTP}] &= \frac{[\text{GTP}_{\text{tot}}^{\text{free}}]}{1 + K_5 [\text{Mg}^{2+}]} \\
[\text{GDP}] &= \frac{[\text{GDP}_{\text{tot}}^{\text{free}}]}{1 + K_6 [\text{Mg}^{2+}]} \\
[\text{EGTA}] &= \frac{[\text{EGTA}_{\text{tot}}]}{1 + K_9 [\text{Mg}^{2+}]} \\
[\text{tub}_{\text{Exposed}}] &= \frac{[\text{tub}_{\text{Exposed}}^{\text{tot}}]}{1 + K_1 [\text{GTP}] + K_2 [\text{GDP}] + K_3 K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_4 K_6 [\text{Mg}^{2+}] [\text{GDP}]} \\
[\text{tub}_{\text{Buried}}] &= \frac{[\text{tub}_{\text{Buried}}^{\text{tot}}]}{1 + K_{10} [\text{GTP}] + K_{11} [\text{GDP}] + K_{12} K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_{13} K_6 [\text{Mg}^{2+}] [\text{GDP}]} \\
[\text{Mg}_{\text{tot}}^{2+}] &= [\text{Mg}^{2+}] (1 + K_5 [\text{GTP}] + K_6 [\text{GDP}] + K_3 K_5 [\text{tub}_{\text{Exposed}}] [\text{GTP}] + \\
&\quad K_4 K_6 [\text{tub}_{\text{Exposed}}] [\text{GDP}] + K_{12} K_5 [\text{tub}_{\text{Buried}}] [\text{GTP}] + K_{13} K_6 [\text{tub}_{\text{Buried}}] [\text{GDP}] + K_9 [\text{EGTA}])
\end{aligned} \tag{S43}$$

We numerically solved Equation Set S43 for $[\text{Mg}^{2+}]$, for each experimental condition defined by $[\text{GTP}_{\text{tot}}^{\text{free}}]$, $[\text{GDP}_{\text{tot}}^{\text{free}}]$, $[\text{EGTA}_{\text{tot}}]$, $[\text{Mg}_{\text{tot}}^{2+}]$, and $[\text{tub}_{\text{tot}}] = [\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}]$ (calculated using Equations S8 and S9). The numerical solution was calculated using *fzero* function of the Matlab[®] software. Using the calculated $[\text{Mg}^{2+}]$ we were able to calculate the total GDP-tubulin mole fraction ($\text{tub-GDP}_{\text{tot}}^{\%}$) out of the total tubulin concentration,

using the following set of equations

$$\begin{aligned}
[\text{GTP}] &= \frac{[\text{GTP}_{\text{tot}}^{\text{free}}]}{1 + K_5 [\text{Mg}^{2+}]} \\
[\text{GDP}] &= \frac{[\text{GDP}_{\text{tot}}^{\text{free}}]}{1 + K_6 [\text{Mg}^{2+}]} \\
[\text{tub}_{\text{Exposed}}] &= \frac{[\text{tub}_{\text{Exposed}}^{\text{tot}}]}{1 + K_1 [\text{GTP}] + K_2 [\text{GDP}] + K_3 K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_4 K_6 [\text{Mg}^{2+}] [\text{GDP}]} \\
[\text{tub}_{\text{Buried}}] &= \frac{[\text{tub}_{\text{Buried}}^{\text{tot}}]}{1 + K_{10} [\text{GTP}] + K_{11} [\text{GDP}] + K_{12} K_5 [\text{Mg}^{2+}] [\text{GTP}] + K_{13} K_6 [\text{Mg}^{2+}] [\text{GDP}]} \\
\text{tub-GDP}_{\text{tot}}^{\%} &= \frac{[\text{tub}_{\text{Exposed}} - \text{GDP}] + [\text{tub}_{\text{Exposed}} - \text{GDP} - \text{Mg}^{2+}]}{[\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}]} + \\
&\quad \frac{[\text{tub}_{\text{Buried}} - \text{GDP}] + [\text{tub}_{\text{Buried}} - \text{GDP} - \text{Mg}^{2+}]}{[\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}]} = \\
&\quad \frac{K_2 [\text{tub}_{\text{Exposed}}] [\text{GDP}] + K_4 K_6 [\text{tub}_{\text{Exposed}}] [\text{GDP}] [\text{Mg}^{2+}]}{[\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}]} + \\
&\quad \frac{K_{11} [\text{tub}_{\text{Buried}}] [\text{GDP}] + K_{13} K_6 [\text{tub}_{\text{Buried}}] [\text{GDP}] [\text{Mg}^{2+}]}{[\text{tub}_{\text{Exposed}}^{\text{tot}}] + [\text{tub}_{\text{Buried}}^{\text{tot}}]}
\end{aligned} \tag{S44}$$

The GDP-tubulin mole fraction was fitted to the experimental data by varying K_1 , K_3 , K_4 , K_{10} , K_{11} , K_{12} , and K_{13} , to minimize Equation S45. As was done in the Excluding the Difference Between Tubulin Dimers With Exposed and Buried E-Sites fitting, K_2 , K_5 , K_6 , and K_9 were adopted from previous works,^{11,12} while K_7 and K_8 were calculated according to Equation S23. The best fitted equilibrium constants are presented in Table 3 and the best fitted GDP-tubulin mole fraction curves are presented in Figure 5.

The minimization was done by the *fmincon* function of the Matlab[®] software, using the

following cost function:

Residuals =

$$\frac{1}{L} \sum_{l=1}^L \left(\frac{\text{tub-GDP}_{\text{tot, Measured}}^{\%} \left([\text{GDP/GTP}]_{0,l} \right) - \text{tub-GDP}_{\text{tot, Modeled}}^{\%} \left([\text{GDP/GTP}]_{0,l} \right)}{\sigma_l} \right)^2 \quad (\text{S45})$$

where L is the number of experimental data points, and $\text{tub-GDP}_{\text{tot}}^{\%}$ is the mole fraction of GDP-tubulin (including dimers with buried and exposed E-sites, either bound or unbound to Mg^{2+}) out of the total tubulin content. σ_l is the experimental error of each data point.

Complete Nucleotide and Tubulin Complexes Distributions

Using the best fitted equilibrium constants (Table 3), the concentrations of all reactants and products of reactions 3-11 and 13-18 were computed, using Equation Sets S43 and S44. In Figures S8 - S20, we plot the mole fraction of each substance (with respect to a specific total concentration of species, as indicated in the figures) as a function of the tubulin-free GDP to tubulin-free GTP molar ratio ($[\text{GDP}/\text{GTP}]_0$). The tubulin-free nucleotides may be either bound or unbound to Mg^{2+} ions. The total tubulin concentrations are indicated in the figures. SEC and BE correspond to SEC Protocol and Buffer Exchange protocol, respectively (see Materials and Methods).

Figure S8: Mole fractions (left axes)/concentrations (in mg/mL, right axes) of tubulin (Tub), tubulin bound to GDP or GTP (tub-GDP/tub-GTP), and tubulin bound to both Mg^{2+} and GDP or GTP, with respect to the total tubulin concentration (indicated in the figure), as a function of tubulin-free GDP to tubulin-free GTP molar ratio ($[\text{GDP/GTP}]_0$). The mole fractions are the sum of tubulin complexes with exposed and buried E-sites (based on Equation Sets S43 and S44). The corresponding separated mole fractions are shown in Figure S14. SEC and BE correspond to SEC Protocol and Buffer Exchange protocol, respectively (see Materials and Methods).

Figure S9: Mole fractions/concentrations of tubulin (Tub) and tubulin bound to GDP or GTP (tub-GDP/tub-GTP) both bound and unbound to Mg^{2+} , with respect to the total tubulin concentration (indicated in the figure), as a function of $[\text{GDP/GTP}]_0$. The mole fractions are the sum of tubulin complexes with exposed and buried E-sites (based on Equation Sets S43 and S44). The corresponding separated mole fractions are shown in Figures S8 and S14. SEC and BE correspond to SEC Protocol and Buffer Exchange protocol, respectively (see Materials and Methods). The crossing point between Tub-GTP to Tub-GDP is indicated by a black circle, and the $[\text{GDP/GTP}]_0$ value is indicated. Note that the crossing points were always at mole fraction of 0.5.

Figure S10: Mole fractions/concentrations of the same tubulin species from Figure S8 with respect to the total tubulin concentration (indicated in the figure), as a function of $[\text{GDP/GTP}]_0$. The model in this figure, however, excluded the difference between tubulin dimers with exposed and buried E-sites (Equation Sets S29 and S30, using the corresponding best fitted equilibrium constants from Table 3). Hence, there is no distinction between the two preparation protocols.

Figure S11: Comparing the mole fractions of the tubulin species at low (1 mg/mL, solid curves) and high (36 mg/mL, broken curves) total tubulin concentration. The analysis is taken from Figure S8 (Buffer Exchange protocol), distinguishing between tubulin dimers with exposed and buried E-sites (left pane) and from Figure S10, assuming no tubulin lattices formed (right pane).

Figure S12: Mole fractions of tubulin dimers with exposed E-sites (Ex) out of the tubulin species in Figure S8 with respect to the total tubulin concentration with exposed E-sites (shown in Figure S15), as a function of $[GDP/GTP]_0$. The total tubulin concentration is indicated in each panel.

Figure S13: Mole fractions of buried (B) tubulin dimers within the tubulin species in Figure S8 with respect to the total buried tubulin concentration (shown in Figure S15), as a function of $[\text{GDP}/\text{GTP}]_0$. The total tubulin concentration is indicated in each panel.

Figure S14: Comparing the mole fractions of tubulin dimers with exposed E-sites (Ex, Figure S12) with the mole fractions of dimers with buried E-sites (B, Figure S13) with respect to the total tubulin concentration (indicated in the figure), as a function of $[GDP/GTP]_0$.

Figure S15: The total mole fraction of tubulin species with exposed (Ex) and buried (B) E-sites with respect to the total tubulin concentration (indicated in the figure), as a function of $[GDP/GTP]_0$. The ratio between tubulin dimers with exposed and buried E-sites was calculated according to Equations S8 and S9, using the free energies detailed in Standard Helmholtz Association Free Energies.

Figure S16: Mole fractions of Mg^{2+} complexes with respect to the total Mg^{2+} concentration (1 mM), as a function of $[\text{GDP}/\text{GTP}]_0$. The results are based on Equation Sets S43 and S44 and the equilibrium constants in Table 3, including the difference between tubulin dimers with exposed and buried E-sites.

Figure S17: Mole fractions/concentrations of tubulin-free GTP complexes with respect to the total added tubulin-free nucleotide concentration (4 mM), as a function of $[GDP/GTP]_0$. The results are based on Equation Sets S43 and S44 and the equilibrium constants in Table 3, including the difference between tubulin dimers with exposed and buried E-sites.

Figure S18: Mole fractions of tubulin-free GTP complexes with respect to the total tubulin-free GTP concentration, as a function of $[\text{GDP}/\text{GTP}]_0$. The results are based on Equation Sets S43 and S44 and the equilibrium constants in Table 3, including the difference between tubulin dimers with exposed and buried E-sites.

Figure S19: Mole fractions/concentrations of tubulin-free GDP complexes with respect to the total tubulin-free nucleotide concentration (4 mM), as a function of $[\text{GDP/GTP}]_0$. The results are based on Equation Sets S43 and S44 and the equilibrium constants in Table 3, including the difference between tubulin dimers with exposed and buried E-sites.

Figure S20: Mole fractions of tubulin-free GDP complexes with respect to the total tubulin-free GDP concentration, as a function of $[\text{GDP/GTP}]_0$. The results are based on Equation Sets S43 and S44 and the equilibrium constants in Table 3, including the difference between tubulin dimers with exposed and buried E-sites.

Nucleotide Content Bound to the Tubulin and Free in the Buffer at Steady-State

To obtain the molar ratio between GTP and GDP at the tubulin E-site, we used the measured total tubulin bound GTP fraction and assumed that the bound nucleotide at the N-site was always GTP. The total tubulin bound GTP mole fraction was subtracted by 0.5, to account for the N-site nucleotide. The remaining fraction was multiplied by 2 to produce GTP mole fractions at the E-site. This multiplication increased the variation between the measured values by a factor of 2. Therefore, the experimental error on the total tubulin bound GTP was only half of the variation computed for the E-site occupancy, shown in Tables 2, S1, and S2, and Figure S1. However, even after multiplication by 2, the variation was small. The measurement error of the buffer nucleotide ratio is similar to the measurement error observed in the purchased nucleotide samples (see Figure S1).

Table S1: Nucleotide content bound to the tubulin dimers (and E-site) and free in the buffer. Fresh tubulin samples () were diluted in BRB80 to the required tubulin concentrations. The samples were then buffer exchanged twice for BRB80, supplemented with the required nucleotide composition, and incubated at 4 °C for 1.5-2 hours. The GTP to GDP molar ratio was simultaneously measured by HPLC on the tubulin dimer and in the buffer. The error was approximated from duplicated measurements.

Tubulin Con- centration mg/mL	Nucleotide Composition	% GTP in buffer	% GDP in buffer	% Total GTP-bound to the tubulin (E-site)	% Total GDP-bound to the tubulin (E-site)
36 ± 2	4 mM GTP	93.2 ± 0.1	6.8 ± 0.1	94.9 ± 0.1 (89.9 ± 0.2)	5.1 ± 0.1 (10.1 ± 0.2)
36 ± 2	2 mM GTP and 2 mM GDP	49.7 ± 0.4	50.3 ± 0.4	74.3 ± 0.3 (48.5 ± 0.5)	25.7 ± 0.3 (51.5 ± 0.5)
36 ± 2	4 mM GDP	4 ± 2	96 ± 2	52.7 ± 0.1 (5.5 ± 0.2)	47.3 ± 0.1 (94.5 ± 0.2)
6 ± 0.3	4 mM GTP	93.2 ± 0.2	6.8 ± 0.2	96.8 ± 0.1 (93.6 ± 0.1)	3.2 ± 0.1 (6.4 ± 0.1)
6 ± 0.3	2 mM GTP and 2 mM GDP	49 ± 2	51 ± 2	79.5 ± 0.2 (59.1 ± 0.3)	20.5 ± 0.2 (40.9 ± 0.3)
6 ± 0.3	4 mM GDP	1.1 ± 0.1	98.9 ± 0.1	55.0 ± 0.1 (10.1 ± 0.1)	45.0 ± 0.1 (89.9 ± 0.1)
1.0 ± 0.1	4 mM GTP	93.5 ± 0.1	6.5 ± 0.1	97.8 ± 0.3 (95.6 ± 0.5)	2.2 ± 0.3 (4.4 ± 0.5)
1.0 ± 0.1	2 mM GTP and 2 mM GDP	47.8 ± 0.5	52.2 ± 0.5	87.5 ± 0.2 (75.1 ± 0.4)	12.5 ± 0.2 (24.9 ± 0.4)
1.0 ± 0.1	4 mM GDP	1.1 ± 0.1	99.0 ± 0.1	59 ± 3 (18 ± 5)	41 ± 3 (82 ± 5)

Table S2: Nucleotide content bound to the tubulin and in the buffer, measured by HPLC. Tubulin samples were eluted from SEC in BRB80, supplemented with 1 mM GTP, and diluted in the SEC running buffer to the required tubulin concentrations. GTP and GDP were then added, and the samples were incubated for 1.5-2 hours. The GTP to GDP molar ratio was simultaneously measured on the tubulin dimer and in the buffer. Error was approximated from duplicated experiments.

Tubulin Concentration mg/mL	Nucleotide Composition	% GTP in buffer	% GDP in buffer	% Total GTP-bound to the tubulin (E-site)	% Total GDP-bound to the tubulin (E-site)
6 ± 0.3	4 mM GTP	93.7 ± 0.1	6.3 ± 0.1	97.0 ± 0.1 (93.9 ± 0.3)	3.0 ± 0.1 (6.1 ± 0.3)
6 ± 0.3	2 mM GTP and 2 mM GDP	51.1 ± 0.1	48.9 ± 0.1	79 ± 2 (57 ± 4)	21 ± 2 (43 ± 4)
2 ± 0.1	4 mM GTP	93.8 ± 0.1	6.2 ± 0.1	98.6 ± 0.4 (97.2 ± 0.8)	1.4 ± 0.4 (2.8 ± 0.8)
2 ± 0.1	2 mM GTP and 2 mM GDP	47.1 ± 0.3	52.9 ± 0.3	86.9 ± 0.5 (73.8 ± 1.0)	13.1 ± 0.5 (26.2 ± 1.0)
1 ± 0.1	4 mM GTP	93.8 ± 0.1	6.2 ± 0.1	98.6 ± 0.2 (97.2 ± 0.3)	1.4 ± 0.2 (2.8 ± 0.3)
1 ± 0.1	2 mM GTP and 2 mM GDP	46.8 ± 0.1	53.2 ± 0.1	88.4 ± 0.5 (76.8 ± 1.0)	11.6 ± 0.5 (23.2 ± 1.0)

Mole Percent of GTP at Onset of the Hydrolysis Kinetics

The mole percent of GTP out of the total nucleotide concentration at the onset of GTP hydrolysis kinetics, in the absence and presence of tubulin are shown in Tables S3 and S4, respectively.

Table S3: The mole percent of total GTP concentration out of the total nucleotide concentration, as measured by HPLC, at the onset of the GTP hydrolysis kinetics in the absence of tubulin, for the data shown in Figure 3.

Temperature	% GTP
-80°C	95.5 ± 0.1
-20°C	95.5 ± 0.1
4°C	95.4 ± 0.1
8°C	95.3 ± 0.1
36°C	95.4 ± 0.1

Table S4: The mole percent of GTP (both bound and unbound to tubulin) out of the total nucleotide concentration, as measured by HPLC, at the onset of the GTP hydrolysis kinetics in the presence of tubulin, for the data shown in Figure 4. Samples were kept on ice and measured at 4°C .

Tubulin concentration	% GTP
$1 \pm 0.1 \text{ mg/mL}$	94.2 ± 0.1
$5.2 \pm 0.2 \text{ mg/mL}$	93.6 ± 0.1
$6.3 \pm 0.3 \text{ mg/mL}$	94.5 ± 0.1

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