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Dynamics of the microtubule oscillator: role of nucleotides and tubulin-MAP interactions

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Microtubules can be induced to perform synchronous and periodic cycles of assembly and disassembly at constant temperature. The process depends on GTP hydrolysis. Time-resolved X-ray scattering using synchrotron radiation shows a cyclic interconversion of tubulin subunits, microtubules and oligomers (= short protofilament fragments). Oscillations are correlated with conditions that stabilize polymers and destabilize oligomers, and others of opposite effect. Microtubule stabilizers include GTP, Mg^{2+} or microtubule-associated proteins (MAPs), destabilizers include GDP or elevated ionic strength. K^+ at intracellular concentrations noticeably increases the stability of tubulin-MAP oligomers, in contrast to Na^+. ATP and the non-hydrolyzable analogue AMP-PNP enhance oscillations by mechanisms that are not directly linked to the role of nucleotide hydrolysis in assembly. We propose a mechanism of oscillations that include oligomers as microtubule disassembly products which transiently lock the protein in an unpolymerizable state; this may point to a role of oligomers in controlling microtubule assembly cycles in cells.

Key words: chemical oscillations/microtubules/GTP hydrolysis/synchrotron radiation/tubulin oligomers

Introduction

Microtubule assembly is commonly considered as a nucleation-condensation process, characterized by a slow nucleation step followed by the endwise addition of tubulin subunits to the growing polymers. This implies pseudo-first order assembly kinetics leading to a steady state (Gaskin *et al.*, 1974; Engelborghs *et al.*, 1976; Johnson and Borisy, 1977). While this model is satisfactory in many conditions, the coupling of GTP hydrolysis to assembly (Weisenberg *et al.*, 1976; Carlier and Pantaloni, 1978) requires additional explanation. The theory was extended to treadmilling (growth of the polymer at one end and shortening at the other at constant polymer length; Margolis and Wilson, 1978) or dynamic instability (coexistence of growing and shrinking polymers at constant polymer mass; Mitchison and Kirschner, 1984). These models showed that nucleotide hydrolysis could cause unexpected dynamical properties. However, in the cases studied the overall assembly progress also took the form of a first order approach to steady state.

The experiments to be described were triggered by earlier observations that tubulin could be driven into a non-equilibrium assembly state, showing overshoot after which the initial population of polymers became unstable, disassembled quickly, and reassembled again in an apparently cyclic fashion that was not expected from existing assembly models (Mandelkow *et al.*, 1983). We then studied the dynamic behavior using a combination of time-resolved X-ray scattering, electron microscopy and biochemical characterization of the nucleotide requirements. One finding was that the conversion from disassembling microtubules to subunits was mediated by oligomers, as seen by cryo-electron microscopy (Mandelkow and Mandelkow, 1985). On the other hand, X-ray scattering showed that there was a temperature-dependent equilibrium between subunits and oligomers that was not directly linked to microtubule assembly (Spann *et al.*, 1987). Both properties play a role in the assembly oscillations. A related study, based on light scattering, was recently reported by Carlier *et al.* (1987).

To provide a framework for the results we recall some key features of microtubule structure and assembly [for recent reviews on microtubule structure and biochemistry see Correia and Williams (1983) and McKeithan and Rosenbaum (1984)]: tubulin subunits (consisting of α-β-heterodimers of tubulin, molecular weight 100 kd) form the basic building blocks; microtubules are made up of 13 protofilaments, each of which consists of a string of subunits running nearly parallel to the tubule axis; and oligomers can be considered as short stretches of protofilaments. Microtubule protein also contains ~ 15-25% microtubule-associated proteins (MAPs) which preferentially bind to oligomers or microtubules. At low temperature tubulin-MAP oligomers often form closed rings. Oligomers used to be considered as initiators of microtubule nucleation; however, their disintegration prior to assembly pointed to a different role (Mandelkow *et al.*, 1980). Microtubule assembly depends on the binding of GTP to tubulin subunits which is hydrolyzed upon polymerization. This nucleotide is rapidly exchangeable on subunits but becomes non-exchangeable in oligomers and microtubules (Jacobs *et al.*, 1974; Zeeberg *et al.*, 1980). By contrast, GDP is a potent inhibitor of assembly and binds to tubulin with a similar affinity as GTP (K_d <0.1 μM). Thus, with regard to the nucleotide status we distinguish (at least) five species, active and inactive subunits (tubulin.GTP and tubulin.GDP), microtubules with GTP and GDP, and oligomers that normally ocur with bound GDP. Other nucleotides can modify the assembly properties in various ways. In particular, microtubule protein contains a nucleoside diphosphate kinase which can generate GTP from protein-bound GDP and added ATP (Jacobs and Huitorel, 1979). Endogenous ATP-dependent kinases can phosphorylate MAPs and tubulin; this weakens the interaction between the proteins and destabilizes microtubules (Jameson *et al.*, 1980; Murthy and Flavin, 1983; Vallee, 1980). Finally, microtubule assembly and the stability of microtubules and oligomers depend strongly on mono- and

This paper is dedicated to Professor K.Wohlfahrth-Bottermann, a pioneer in the study of oscillations in the cytoskeleton, on the occasion of his 65th birthday.

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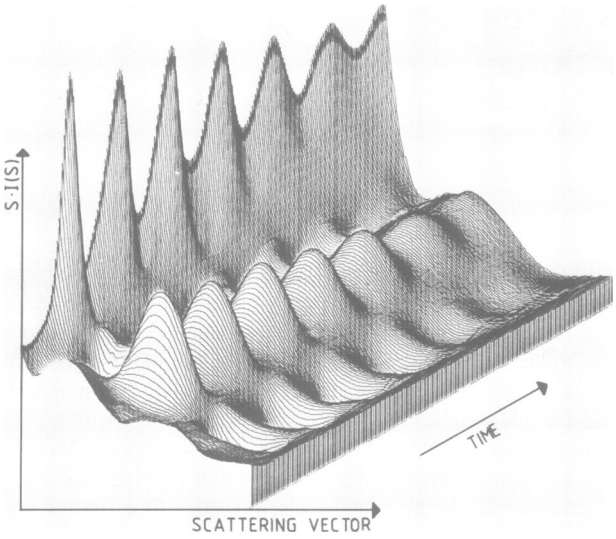

Fig. 1. Time-resolved X-ray scattering from a solution of oscillating microtubules, showing the X-ray intensity (*z*-axis) as a function of scattering angel vector ($S = 2\sin\theta/\text{lambda}$,*x*-axis) and time (*y*-axis, 3 s scan interval). Microtubule protein at 32 mg/ml, assembly buffer with 4 mM GTP, 3 mM AMP−PNP, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 60 mM NaCl. The central scatter (left) indicates overall assembly, the subsidiary maximum arises from microtubules. The arrow marks the initial temperature jump. The periodicity of the fluctuations is ~ 2 min. The final state (after disappearance of the oscillations, not shown) is dominated by the scattering from oligomers.

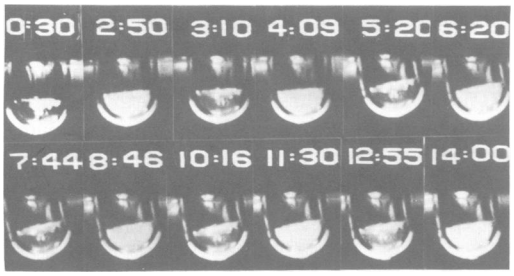

Fig. 2. Light scattering of oscillating microtubule specimens observed by video imaging. The reaction was started by placing the test tube (diameter 5 mm) in a 37°C water bath. The turbidity shows maxima and minima at ~ 2-min intervals. The light scattering signal shown here is roughly comparable to the central scatter of the X-ray experiments but contains no information on the types of structures in solution. PC-tubulin at 42 mg/ml, assembly with 4 mM GTP, 15 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 40 mM NaCl.

divalent cations (Olmsted and Borisy, 1975). All of the above factors that affect microtubule assembly influence the nature of the oscillations. In this paper we describe how the microtubule oscillator is driven by GTP hydrolysis, and how it depends on parameters that alter microtubule stability and tubulin−MAP interactions, such as ionic conditions, nucleotides or phosphorylation. More details on the properties of oligomers and on the mathematical analysis of the oscillations will be given elsewhere.

Results

Structural and kinetic properties of microtubule oscillations

The basic features of the microtubule oscillator are illustrated in Figure 1. Assembly is initiated by a temperature jump from 3 to 37°C (at t = 0), leading to a simultaneous increase of the central scatter (left) which reflects the overall degree of polymerization, and of the side maximum around 0.05 nm^{-1} sensitive to the formation of microtubules. If assembly followed a nucleated condensation mechanism one would expect an exponential approach to the steady state. This is not observed here; instead, the microtubules initially formed become unstable and disassemble autonomously down to 40% of their peak level, even though the temperature is maintained at 37°C. After 50 s the decay is stopped, the solution returns to another round of assembly with subsequent disassembly, and so on. The periodicities are typically in the 1−3 min range. The oscillations are damped out after ~ 13 min; at this point part of the initial microtubules have been replaced by oligomers (notice the reduced side maximum). The final state is dominated by the scattering from tubulin oligomers, not microtubules (not shown in Figure 1, but see Figure 4; for details on the interpretation see Bordas *et al.*, 1983; Spann *et al.*, 1987).

Oscillations can be observed both with purified tubulin (Figures 2 and 3) and microtubule protein (Figures 1 and 4). In both cases one requires fairly high protein concentrations, ~ 10 mg/ml and up. In principle, oscillations can also be observed by eye (Figure 2) or by light scattering (Figure 5). However, the turbidity records only part of the change seen by X-rays and is insensitive to the difference between the various assembly forms. This is due to the long wavelength and limited resolution of visible or UV light so that the data presented below are based on the analysis of the X-ray traces. Note that the final solution in Figure 1 has a high central scatter. For visible or UV light this translates into a high turbidity which is usually taken as an indicator of microtubules, but this interpretation cannot be taken for granted in the presence of oligomers.

The key feature of the oscillations is the periodic reshuffling between the tubulin assembly forms, primarily between microtubules and oligomers; this can be demonstrated by comparing the X-ray traces (Figures 1 and 4), or by monitoring an oscillating sample with electron microscopy. This shows predominantly mirotubules at the assembly peaks (Figure 3a), a large contribution from oligmers at the troughs (Figure 3c) and microtubules disassembling into oligomers during the decay phase (Figure 3b). This is similar to earlier results with cryo-electron microscopy (Mandelkow and Mandelkow, 1985) where cold-induced disassembly was studied in standard buffer conditions.

Role of GTP as the driving force in microtubule assembly and oscillations

Oscillations are only observed with protein that is competent to assemble into microtubules; as a corollary, microtubule inhibitors also inhibit oscillations. Thus, neither assembly nor oscillations occur in the absence of GTP which is required for the activation of tubulin subunits (tubulin.GTP; a typical threshold level is 0.5−1 mM GTP for the conditions of Figure 4). GTP is used up during the oscillations; the consumption is not uniform but correlated with the assembly cycles (Figure 5). Conversely, GDP efficiently inhibits assembly and oscillations, even in the presence of excess GTP, by keeping tubulin in its inactive form (tubulin.GDP) and by stabilizing oligomers (Howard and Timasheff, 1986; Spann *et al.*, 1987). The effect is mainly due to GDP's interference with microtubule nuclea-

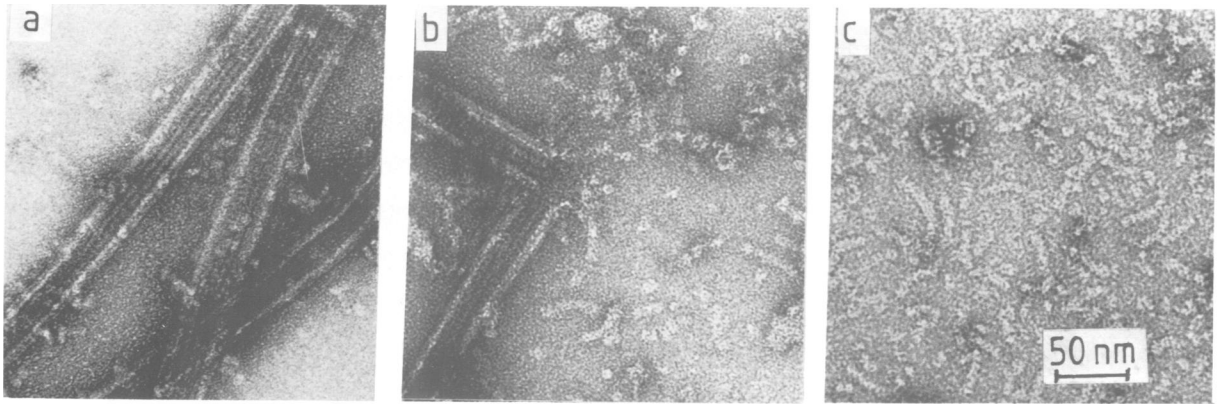

Fig. 3. Elecron micrographs of PC-tubulin at different stages of oscillations (conditions see Figure 2). Left, maximal assembly. One observes microtubules of normal appearance and oligomeric material in the background. Center, phase of disassembly. There is a noticeable increase in oligomers. Many microtubules appear broken. This may occur either during disassembly in solution or as a result of the preparations for electron microscopy (e.g. negative staining or drying), but in any case it indicates a lower stability of the microtubules. Right, field of oligomers at an assembly minimum. Typical lengths are between 20 and 100 nm, corresponding to 3–12 tubulin dimers. The longer ones clearly show the coiling characteristic of disassembled protofilament fragments that leads up to ring-like closure (see upper left). Complete rings are rare with PC-tubulin but frequent with microtubule protein, presumably due to the stabilization of oligomers by MAPs.

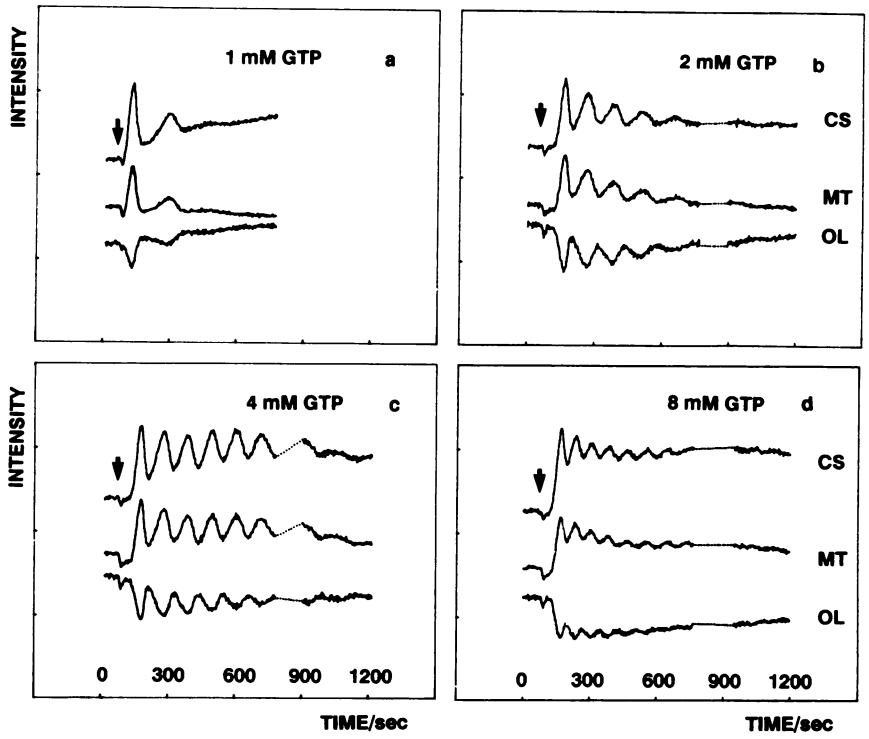

Fig. 4. Dependence of microtubule assembly oscillations on GTP concentration monitored by X-ray scattering at different angles (see Figure 1) as a function of time. Top traces, central scatter (S = 0.02 nm^{-1}) sensitive to overall assembly; bottom traces, region around S = 0.033 nm^{-1} sensitive mainly to rings and smaller oligomers; center traces, region around S = 0.05 nm^{-1} sensitive to the formation of microtubules. The arrow marks the temperature jump from 3 to 37°C. The dotted regions represent breaks caused by data transfer to the computer. Conditions: Microtubule protein at 39 mg/ml (prepared by three cycles of assembly and disassembly in 0.1 M Pipes–NaOH, pH 6.9, with 1 mM ATP). Assembly buffer of experiment is 0.1 M Pipes–NaOH, pH 6.9, with 1 mM each of AMP–PNP, DTT, EGTA, 20 mM $MgCl_2$, 60 mM NaCl and various concentrations of GTP. (**a**) 1 mM GTP. Note rapid overshoot of microtubule assembly and decay, followed by two strongly damped oscillations of ~ 2.5-min periodicity. The subsequent slow increase at low angles is due to nonspecific aggregation and is typical of samples depleted of GTP. (**b**) 2 mM GTP. Five oscillations are visible, mean periodicity is 2 min. (**c**) 4 mM GTP. 11 oscillations are visible (not all shown), strong damping sets in after the sixth cycle. (**d**) 8 mM GTP. Eight oscillations are visible. Note that both the amplitudes and periodicities of the oscillations decrease with increasing concentrations of GTP. No oscillations are observed below 0.5 mM GTP in these conditions. Note that the bottom curves oscillate in antiphase to the top and center curves.

tion, i.e. the inhibition by GDP added before the first assembly is much more pronounced than that of GDP generated through GTP hydrolysis following assembly. Non-hydrolyzable GTP analogues (e.g. GMP–PNP) or slowly hydrolyzable ones (GMP–CPP) support microtubule assembly but not oscillations.

The above points are illustrated in the experiments of Figure 4, performed with microtubule protein in conditions that favor oscillations, i.e. rapid temperature jump, high protein concentration, and elevated levels of Mg^{2+} and monovalent cations. At 1 mM GTP (Figure 4a), the temperature jump leads first to a brief undershoot, followed by a rapid

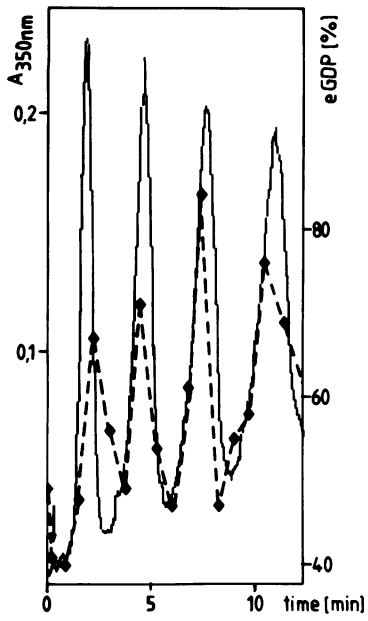

Fig. 5. Oscillations in protein-bound nucleotides. Solid line: Turbidity of an oscillating sample measured by absorption at 350 nm (left scale) in a Beckman DU 40 spectrophotometer, using the same temperature jump cell as in the X-ray experiments (path length 1 mm). The background turbidity was subtracted. Dashed line: GDP bound to the exchangeable nucleotide binding site determined by HPLC (see Materials and methods), normalized with respect to the total concentration of subunits. Conditions: Microtubule protein (42 mg/ml) prepared by 3 cycles in standard assembly buffer (0.1 M Pipes–KOH, pH 6.9, with 1 mM ATP). Assembly buffer of the experiment is 0.1 M Pipes-KOH, pH 6.9, with 1 mM DTT, 1 mM EGTA, 20 mM $MgCl_2$, 60 mM NaCl and 4 mM GTP. The peaks of protein-bound GDP (resulting from GTP hydrolysis) roughly coincide with the peaks of turbidity which correspond to maximum microtubule assembly, as determined from parallel X-ray experiments.

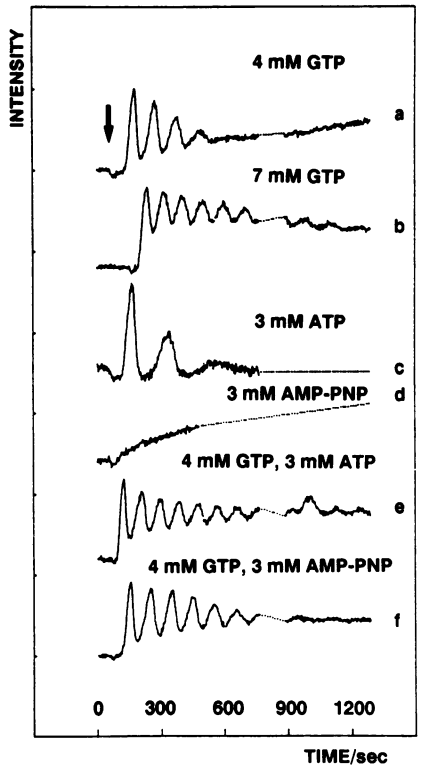

Fig. 6. Influence of GTP, ATP, and AMP–PNP on oscillations. Central scatter, $S = 0.02$ nm^{-1}. Conditions: Microtubule protein at 32 mg/ml (prepared by 3 cycles of assembly and disassembly in 0.1 M Pipes–NaOH pH 6.9 assembly buffer, with 1 mM GTP). Assembly buffer of experiment is 0.1 M Pipes–NaOH, pH 6.9, with 1 mM DTT, 1 mM EGTA, 20 mM $MgCl_2$, 60 mM NaCl and various concentrations of nucleotides. (**a**) 4 mM GTP. Four oscillations and subsequent slow non-specific aggregation; periodicity 1.6 min. Note the smaller number of cycles, compared to Figure 4b. (**b**) 7 mM GTP. Six oscillations with little damping, followed by four strongly damped ones. As in Figure 4, the amplitudes and periodicities decrease with increasing GTP. (**c**) 3 mM ATP. Only three slow oscillations can be seen clearly. In this case assembly depends on the generation of GTP via the endogenous NDP kinase. (**d**) 3 mM AMP–PNP. There is no microtubule assembly. After the temperature jump one only observes a small undershoot and a slow increase due to nonspecific aggregation common in the absence of GTP. (**e**) 4 mM GTP and 3 mM ATP. There are nine clear oscillations; note the onset of strong damping after the 9th cycle. (**f**) 4 mM GTP nd 3 mM AMP–PNP. Seven weakly damped oscillations, with strong damping thereafter. The periodicities are similar to Figure 6e, but the amplitudes are even more enhanced. This experiment shows that the improvement of oscillations of microtubule protein at higher nucleotide concentrations cannot simply be explained by the direct involvement of GTP in microtubule assembly.

overshoot, two more strongly damped cycles, and a slow increase in the scattering at low angles. The undershoot is due to the dissociation of oligomers initially present (the association of tubulin into oligomers is exothermic, see Spann *et al.*, 1987; for a more dramatic undershoot see Figure 8e). The rise of the central scatter (sensitive to overall assembly, top curve) and at $S = 0.05$ nm^{-1} (sensitive to microtubules, center curve), combined with the drop at $S = 0.033$ nm^{-1} (sensitive to oligomers, bottom curve) indicates the rapid formation of microtubules (compare Figure 1). After the first maximum ~ 90% of the microtubule mass disappears again. During the subsequent oscillations part of the microtubules are recovered, superimposed on a rising level of oligomers. After the oscillations are damped out the solution contains largely oligomers; the low angle scatter (top and bottom curves) continues to rise because of nonspecific aggregation which is typical of solutions depleted of GTP. The overshoot behavior shown here is characteristic of situations where factors necessary for assembly transiently drop to a low level, thus limiting the supply of active subunits.

Figure 4a–d show the effects of rising GTP concentrations up to 8 mM. The number of oscillations visible above noise increases, they become faster (i.e. the mean periodicity decreases from 2.5 to 1 min), the amplitudes decrease, whereas the initial maximum of assembly remains constant. Note that the damping of the oscillations is not uniform; the number of cycles damped only weakly increases with the initial GTP. In parallel experiments we determined the level of GTP and GDP bound to the protein (Figure 5). Both showed oscillations that were correlated with the buildup and decay of microtubules. Protein-bound GDP reached maxima at or slightly after the assembly peaks, reflecting the greater number of subunits locked in the polymer state. The peaks of protein-bound GTP coincided roughly with the troughs between assembly, consistent with an increase in the tubulin pool. Thus the consumption of GTP occurs in bursts coupled to assembly cycles (due to the incorporation of tubulin.GTP into microtubules and subsequent hydrolysis of GTP). It appears that the onset of strong damping can be correlated with a threshold ratio of GDP to GTP.

The oscillations usually cease at a point where microtubules are still present and coexist with oligomers. With

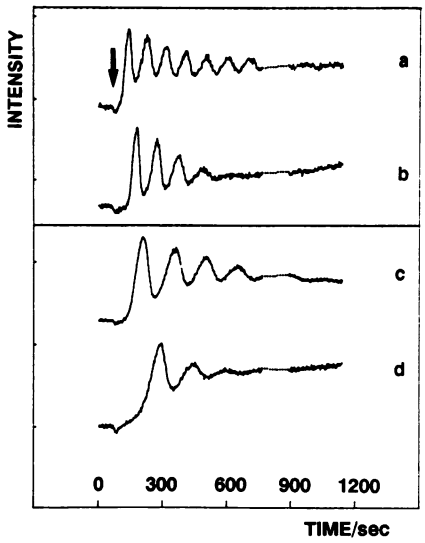

Fig. 7. Influence of cycle preparation in ATP (enhancing phosphorylation by endogenous kinases) on oscillations. Central scatter, $S = 0.02\ nm^{-1}$. (**a**) and (**b**), microtubule protein (30 mg/ml) prepared by three cycles of assembly and disassembly in 0.1 M Pipes−NaOH, pH 6.9, with 1 mM ATP (**a**) or 1 mM GTP (**b**). Assembly buffer of experiment is 0.1 M Pipes, pH 6.9, with 1 mM each of DTT and EGTA, 20 mM $MgCl_2$, 60 mM NaCl and 4 mM GTP. Note the more extended oscillations in the ATP-cycled sample (**a**). (**c**) and (**d**), PC-tubulin at 35 mg/ml prepared by three cycles of assembly/disassembly in standard buffer containing either 1 mM ATP (**c**) or 1 mM GTP (**d**), and then purified by phosphocellulose chromatography (see Materials and methods). Assembly buffer of experiment is 0.1 M Pipes, pH 6.9, with 1 mM DTT, 1 mM EGTA, 20 mM $MgCl_2$, 60 mM NaCl, 4 mM GTP and 3 mM AMP−PNP. Oscillations are more pronounced in sample (**c**), prepared by cycles in ATP.

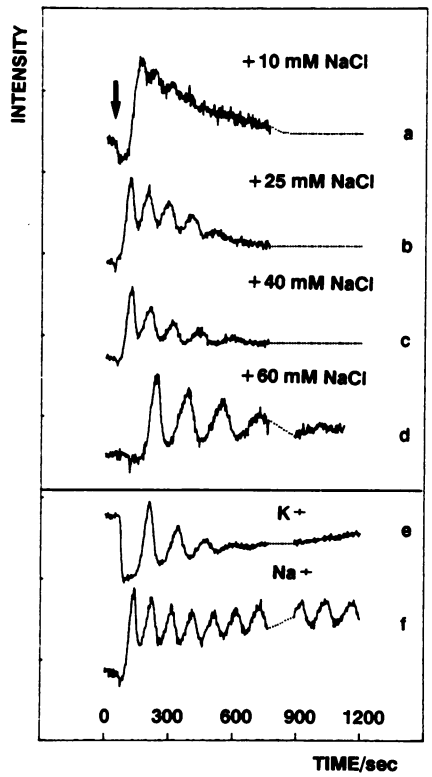

Fig. 8. Dependence of oscillations on monovalent cations. Central scatter, $S = 0.02\ nm^{-1}$. (**a**)−(**d**), influence of Na^+. Thrice cycled microtubule protein in standard assembly buffer. Assembly buffer of experiment is 0.1 M Pipes−NaOH, pH 6.9, with 1 mM DTT, 1 mM EGTA, 15 mM $MgCl_2$, 2 mM GTP and additions of NaCl as listed. (**a**) +10 mM NaCl. There are a few weak oscillations superimposed on a slow decay due to GTP consumption. Microtubule assembly is preceded by a clear undershoot and slow nucleation. At the end of the experiment oligomers dominate the pattern, as judged from the full scattering pattern (not shown). (**b**) +25 mM NaCl. Five oscillations are clearly visible, initial assembly becomes more rapid than in (**a**). As before the final state consists of mainly oligomers. (**c**) +40 mM NaCl. Oscillations are similar to (**b**), but the amplitudes become more pronounced. (**d**) +60 mM NaCl. Amplitudes are further increased (compare **c**), but oscillations become slower. (**e**)−(**f**): Comparison between Na^+ and K^+. The protein was prepared by three cycles of asembly/disassembly with 0.1 M Pipes−KOH and 1 mM ATP. In (**e**) the protein was adjusted to the assembly buffer conditions of the experiment at a concentration of 30 mg/ml. In (**f**) the Pipes−KOH buffer was exchanged to Pipes−NaOH over a Pharmacia PD10 column and then adjusted to the same protein concentration and assembly conditions as in (**e**). The assembly buffer of both experiments was 0.1 M Pipes, pH 6.9, with 1 mM each of DTT and EGTA, 20 mM $MgCl_2$, 60 mM NaCl and 4 mM GTP. Thus specimen (**e**) contains ~ 160 mM K^+ (from adjustment of 0.1 M Pipes at pH 6.9) and 60 mM Na^+, while specimen (**f**) contains a total of ~ 220 mM Na^+. Note that in these conditions elevated K^+ (**e**) leads to an enhanced stability of oligomers, best seen from the high initial scattering level and the dramatic undershoot prior to the first microtubule assembly; this correlates with a reduced capacity for oscillations. In Na^+ (**f**) the oligomer stability is reduced, and the oscillatory behavior is enhanced (compare **b**−**d**).

time, microtubules disappear in an apparently continuous fashion, the final state being dominated by oligomers. The life time of microtubules can be increased by a GTP regenerating enzyme system (e.g. acetate kinase and acetyl phosphate). However, this leads only to a moderate improvement of the oscillations (not shown).

Influence of nucleotides on oscillations

Preparations of microtubule protein based on cycles of assembly and disassembly can be made using either GTP or ATP. This is due to a copurifying nucleoside diphosphate kinase capable of regenerating GTP from GDP, using ATP as a phosphate donor. This enzyme can be inhibited by the non-hydrolyzable ATP analogue, AMP−PNP. In the course of varying nucleotide conditions we noticed that they modify microtubule assembly and oscillations in ways that go beyond the regeneration of GTP. This is best understood in terms of the stability of microtubules and their interactions with MAPs, as described below.

Figure 6a and b show a sample of microtubule protein at two different GTP concentrations, 4 and 7 mM, reproducing essentially the behavior described in Figure 4, e.g. more and faster oscillations at higher GTP concentrations. Figure 6c was obtained with 3 mM ATP. This curve is reminiscent of assembly with low GTP (e.g. Figure 4a) and can be explained by the limited regeneration of GTP via the NDP kinase; as a control, no assembly takes place with 3 mM of the inhibitor AMP−PNP (Figure 6d). When the experiment is done with 4 mM GTP and 3 mM ATP (Figure 6e), the oscillations become surprisingly enhanced, compared with 4 mM GTP alone (see Figure 6a). The magnitude of this effect cannot be accounted for simply by the regeneration of GTP by the added ATP. In fact, a similar increase can be achieved using 4 mM GTP and 3 mM AMP−PNP (Figure 6f). This enhancement must therefore be due to a generalized effect of nucleotides that is not directly related to the production of GTP and its involvement in microtubule assembly.

As a control, we tested this behavior in related experiments with purified tubulin (such as in Figure 2 or 3; details elsewhere). This protein shows similar oscillations, driven by GTP hydrolysis, but the generalized nucleotide effect is reduced. It appears therefore that this feature resides partly in the MAPs and probably results from a decreased affinity between MAPs and microtubules and/or oligomers (see Discussion).

In another set of experiments we tested the effect of replacing GTP by ATP during the first cycles of microtubule protein preparation. Pairs of protein samples were treated identically except that one had 1 mM ATP in the first three purification cycles (Figure 7a,c), the other 1 mM GTP (Figure 7b,d). The subsequent experiments were performed with GTP in both cases. Figure 7a and b show the behavior of microtubule protein cycled initially with ATP or GTP, Figure 7c and d are the corresponding experiments with PC-tubulin. In both cases the oscillations are strongly enhanced in the ATP-treated samples. This may be related to the phosphorylation of the protein by ATP-dependent endogenous kinases and the resulting decrease in the tubulin−MAP and tubulin−tubulin interactions (Jameson *et al.*, 1980), as discussed below.

Modulation of oscillations by ionic conditions

We now describe experiments originally designed to test the hypothesis that tubulin−MAP interactions are important for the magnitude and duration of the oscillations. Elevated monovalent salts are known to reduce the affinity of MAPs for tubulin oligomers or microtubules, thereby altering their stabilities (Marcum and Borisy, 1978; Vallee, 1982).

In Figure 8a−d we compare the effect of rising Na^+ on the oscillations in the presence of 2 mM GTP and 15 mM Mg^{2+}. The buffer itself (0.1 M Pipes) contains an initial concentration of ~ 160 mM Na^+ due to the pH adjustment with NaOH, so that the effective variation is from 170 to 220 mM Na^+. At the lower concentrations we observe the usual prenucleation events, followed by assembly with a few weak oscillations, superimposed on a slow decay due to GTP consumption. The final solution contains largely oligomers. The nature of the counterion (Cl^- or SO_4^-) has no noticeable influence. A further rise in Na^+ strongly promotes the oscillations and shows a broad optimum around 200 mM. The initial assembly becomes faster, the amplitudes increase and the periodicities decrease. At even higher concentrations of Na^+ assembly becomes suppressed altogether. The optimum of oscillations occurs at higher salt concentrations than the optimum of assembly in standard conditions (see Olmsted and Borisy, 1975). The shift is related to the higher Mg^{2+} concentrations (15 mM instead of the standard 1 mM) which are known to favor tubulin self-assembly (Frigon and Timasheff, 1975; Herzog and Weber, 1977). These experiments illustrate how oscillations are related to two antagonistic effects, stabilization of tubulin assemblies (by Mg^{2+}) and destabilization of the tubulin−MAP interaction via Na^+. The combination of factors that will optimize the oscillations depends on other factors as well, particularly protein concentration and phosphorylation (see below).

That the above salt effect is not due to ionic strength alone can be demonstrated by comparing Na^+ with K^+, the cation present within cells. Figure 8e and f show two microtubule protein samples prepared identically by the temperature cycle procedure, using 0.1 M Pipes, pH 6.9 adjusted with KOH and 1 mM ATP in the first three cycles. In this buffer the concentration of the monovalent counterion K^+ is ~ 160 mM. Prior to the experiment the pellet of sample (e) was redissolved in the assembly buffer given in the legend, containing 4 mM GTP, 20 mM Mg^{2+} and 60 mM Na^+ so that the total concentration of monovalent cations was 220 mM. In sample (f) the buffer of the protein was exchanged over a column, thereby replacing all K^+ ions by Na^+, giving a total of 220 mM Na^+ and the other factors as before. This comparison was triggered by our earlier observation (Spann *et al.*, 1987) that buffers containing mainly K^+ stabilized oligomers and therefore showed a higher central scatter at low temperature, with a corresponding larger undershoot after the temperature jump due to the dissolution of the oligomers. We therefore use the ionic composition as a means to probe the role of oligomer stability in assembly and oscillations.

As expected, the microtubule protein in K^+/Na^+ (Figure 8e) shows a high initial scatter, large undershoot after the T-jump, followed by slow nucleation, rapid assembly, four slow and strongly damped oscillations with a large initial amplitude (90%) and finally the usual slow degradation due to depletion of GTP (note that with 1 mM Mg^{2+} and without the added Na^+ the assembly would reach a stable plateau without oscillations). By contrast, the sample containing Na^+ only (f) shows a low initial scatter, small undershoot and 13 rapid and weakly damped oscillations whose amplitude is smaller than in (a) (50%). These experiments suggest that factors that stabilize oligomers (such as K^+) tend to reduce the protein's capacity to oscillate. The difference between the counterions is reduced with purified tubulin, suggesting that the stabilizing effect of K^+ is partly

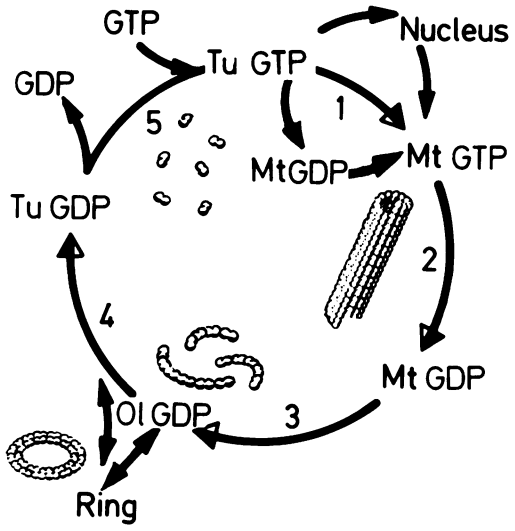

Fig. 9. Model of reaction cycle responsible for oscillations. Microtubules (Mt.GTP) are formed from active subunits (Tub.GTP, step 1), GTP is hydrolyzed upon assembly (MtD, step 2), leading to disassembly of microtubules into oligomers loaded with GDP (Olig.GDP) which transiently lock the subunits in an unpolymerizable state. When inactive subunits (Tub.GDP) are released from oligomers (step 4) they can be recharged to Tub.GTP (step 5), leading to the reassembly of microtubules. Side reactions are the dissolution of rings into oligomers and subunits (observed with microtubule proteins just after the T-jump and responsible for the undershoot, see e.g. Figure 8a and e), and the nucleation during the first round of assembly.

due to the tubulin−MAP interaction, or, conversely, that Na^+ is more efficient in weakening that interaction.

Discussion

Model reaction mechanism for microtubule oscillations

The structural and kinetic properties of the microtubule oscillator can be explained by the reaction cycle of Figure 9. Starting the cycle at step 1, the assembly-competent ('active') subunits tubulin. GTP form microtubules. If this takes place *de novo*, e.g. after the temperature jump to 37°C, one requires an intermediate nucleation step, but if microtubules are already present, subunits can simply add to the ends (endwise elongation). Once the subunits are incorporated, GTP is hydrolyzed (step 2). This destabilizes the microtubules, and when the saturation with GDP reaches some threshold they begin to disassemble into oligomers (short protofilament fragments, step 3), preferentially from their ends, but to some extent also by internal breakage. Oligomers with bound GDP cannot be recharged with GTP, but they disintegrate into inactive tubulin.GDP subunits (step 4) which can be recharged (step 5) as long as GTP is available in solution. The reactivated subunits are then added back to microtubules (step 1), closing the cycle. A side reaction is the disintegration of ring oligmers into smaller fragments and subunits, observed with microtubule protein just after the temperature jump. Notice that this scheme reduces to the common reaction for polymer elongation, microtubules + subunits ⇌ microtubules, in conditions where the disintegration of oligomers into subunits is rapid.

What evidence is available for this model? The activation of tubulin for assembly by GTP and the inhibition by GDP have been described by many authors (e.g. Weisenberg *et al.*, 1976; Carlier and Pantaloni, 1978). Nucleotides can be exchanged only on dispersed tubulin subunits, but neither on microtubules nor on oligomers (Jacobs *et al.*, 1974; Zeeberg *et al.*, 1980; A.Jagla *et al.*, in preparation). X-ray studies have shown that oligomers disintegrate prior to assembly (Mandelkow *et al.*, 1980), and they are in a temperature-dependent equilibrium with subunits (Spann *et al.*, 1987). Finally, depolymerization of microtubules into oligomers can be demonstrated even in non-oscillatory conditions by time-resolved cryo-electron microscopy (Mandelkow and Mandelkow, 1985).

The basic mechanism applies to tubulin with or without MAPs. When MAPs are present (as in microtubule protein) they associate preferentially with microtubules and oligomers (leading to their ring-like closure; Weingarten *et al.*, 1975; Vallee and Borisy, 1978). MAPs stabilize oligomers and microtubules, even to the extent of making them metastable in excess GDP. Thus, factors that reduce the tubulin−MAP affinity destabilize microtubules and oligomers. One example is high ionic strength which can be used to separate MAPs from microtubules (Vallee, 1982). Another one is the phosphorylation of MAPs and/or tubulin. This can be achieved by a variety of kinases, in particular by the ATP-dependent endogenous kinases present in cycled microtubule protein (see Jameson *et al.*, 1980; Sloboda *et al.*, 1975). This probably explains the enhancement of oscillations prepared by the cycle procedure in the presence of ATP (Figure 7).

The above assembly scheme contains the autocatalytic reactions that are in general necessary for producing oscillations, and it shows how the cycle is driven by GTP hydrolysis. However, the presence of these steps does not guarantee oscillations. Whether the system shows a first-order approach to steady state (the usual case studied in many earlier reports) or develops oscillations, as in the present case, depends on particular combinations of rate constants which in turn depend on chemical and physical conditions. We are at present investigating the requirements by computer simulation (to be described elsewhere), but we note a few important conditions here: a rapid temperature jump (causing a high nucleation rate), high protein concentration (favoring oligomerization), an appreciable difference in the stability of microtubules before and after GTP hydrolysis (this initiates disassembly), weak or no stabilization of microtubules by MAPs (this can be reduced by high salt, phosphorylation, etc.), a sufficiently high stability of oligomers (capable of withdrawing tubulin from the pool of repolymerizable active subunits) and, of course, an energy source in the form of GTP.

Given the number of variables it is clear that oscillatory behavior can be approached from various directions. At our highest concentrations of tubulin dimers (up to 50 mg/ml PC-tubulin) we obtain oscillations in standard assembly buffer, i.e. without elevated salts or Mg^{2+}. In this buffer the dimer concentration can be dropped down to ~ 10 mg/ml when the protein has previously been cycled in ATP and thus is presumably phosphorylated. In general, oscillations can be induced by balancing microtubule stabilizers (e.g. Mg^{2+}) and destabilizers (e.g. high ionic strength). This balance also seems to underly the enhancing effect of high nucleotide concentrations which is distinct from GTP's direct involvement in polymerization. This would be consistent with earlier reports showing that microtubule assembly can be inhibited by high GTP or ATP (Olmsted and Borisy, 1975; Jameson and Caplow, 1980) and that ATP can bind weakly to tubulin at a site different from GTP (Zabrecky and Cole, 1982).

In most cases the oscillations stop before GTP is exhausted, showing that the energy supply alone is not sufficient for non-equilibrium behavior. This points to the importance of an initial synchronization of the solution which can be achieved, for example, by a rapid temperature jump and at high protein concentrations.

Relationship with models of microtubule assembly and dynamics

Following the original treatment developed for actin assembly (Oosawa and Kasai, 1962), most authors described microtubule assembly in terms of nucleation, followed by endwise association of subunits of the type microtubules + subunits ⇌ microtubules. Assuming that *de novo* nucleation stops at an early stage this reaction implies a pseudo-first order approach to equilibrium where a critical concentration of subunits remains in solution (Gaskin *et al.*, 1974; Engelborghs *et al.*, 1976; Johnson and Borisy, 1977), but there is no room for oscillations. Further extensions of the model dealt with the problem that GTP was required for assembly but was hydrolyzed thereafter. In the treadmilling model (Margolis and Wilson, 1978) a distinction was made between the two polymer ends (one growing, one shrinking at constant overall length). In the dynamic instability model (Mitchison and Kirschner, 1984) the microtubules were subdivided into two populations (one growing, one shrinking, without change in overall polymerization).

This model was linked to the GTP cap hypothesis (Carlier *et al.*, 1984) which postulated that microtubule ends covered with tubulin.GTP subunits were stable and capable of growing, while those with tubulin.GDP ends disassembled. Both models assumed an approach to steady state of the nucleation−condensation type and specifically dealt only with situations where the assembled polymer mass remained constant. These schemes therefore do not account for oscillations of the type observed here. However, indications that microtubule assembly could be even more complex came from Horio and Hotani's (1986) images of single microtubules which showed length fluctuations of both ends that were of unequal magnitude and uncorrelated in time. Recently, oscillations in bulk samples were studied by light scattering (Carlier *et al.*, 1987). With regard to microtubule assembly the results of that method are comparable to the low angle region of the X-ray scattering, and the basic properties of the oscillations described here are similar to those of Carlier and co-workers. These authors discuss a computer simulation which produces oscillations assuming the presence of a GTP cap on the ends of microtubules. It should be noted, however, that other models are also capable of generating oscillations.

The main difference between X-ray scattering and other methods is that the former is sensitive to species other than microtubules, such as oligomers which are difficult to detect by light scattering because of their small size. In standard assembly conditions (leading to first order assembly kinetics and a stable steady state) the oligomers make only a minor contribution to the X-ray pattern. However, they become pronounced in oscillatory conditions, and their level varies in antiphase with microtubules. Moreover, the GDP bound to oligomers is non-exchangeable, in contrast to tubulin subunits where the nucleotide exchange is rapid (details to be described elsewhere). These features, combined with the oligomers seen on disassembling microtubules observed by cryo-electron microscopy have lead to the model of Figure 9.

Thus our reaction mechanism differs from previous ones in that oligomers are viewed as intermediates between microtubules and subunits in microtubule disassembly. This is in contrast to earlier studies in which oligomers were either disregarded or viewed as intermediates of assembly. Oligomers represent a second state, besides microtubules, where the subunits are transiently locked and thus removed from the subunit pool. This allows microtubules to disassemble after GTP hydrolysis without the immediate reactivation of the released subunits; this can only occur after a time lag depending on the stability of the oligomers. In other words, oligomers act as 'slow release' tubulin storage and delivery buffer. The transient removal of tubulin subunits from the solution is possible even in excess GTP since the nucleotides bound to oligomers are non-exchangeable.

A unique feature of the oscillations is the correlated behavior of microtubules. If growth and shrinkage did not occur simultaneously no net oscillations would be observed. The initial trigger comes from the temperature jump; however, after that the synchronization must be maintained by chemical signalling, using reactions such as those listed in the above scheme. It is possible that fluctuations of individual microtubules continue beyond the point where bulk oscillations become invisible. This would be due to the loss of synchronization rather than to loss of dynamic behavior. Finally, we mention that in most cases microtubules do not completely disappear at the troughs between assembly peaks. Regrowth could therefore take place by elongation of the existing microtubules and would not require new nucleation, although the latter process could contribute as well.

The proposed reaction scheme contains the basic features known from other biochemical oscillators, e.g. the need for an external energy source and autocatalytic steps (see Hess and Boiteux, 1971). One property which distinguishes the microtubule system from others is the coupling of a chemical reaction cycle to the periodic construction and destruction of a polymer. It can therefore be observed by structural methods (X-ray scattering) and be correlated with the biochemical status of the protein (nucleotide binding). Another unique feature is the periodic change of the diffusion constant of tubulin. This point does not follow explicitly from the reaction equations, but it is physically clear from the fact that the subunits become immobilized in the polymer and, to a lesser extent, in the oligomer.

Although oscillations in microtubule assembly reported here occur *in vitro* they are a well-known part of the cell cycle in eukaryotic cells. This takes place on a different time scale and is presumably governed by an elaborate control mechanism. Nevertheless, the fact that tubulin can mimick these events by itself suggests that the underlying molecular interactions may be operational in cells. This could hold in particular for tubulin oligomers which act as transient storage aggregates and thereby control the availability of polymerizable tubulin.

Materials and methods

Protein preparation

Microtubule protein and PC-tubulin was prepared by three temperature-dependent cycles of assembly and disassembly as described (Mandelkow *et al.*, 1985). The standard reassembly buffer was 0.1 M Pipes, pH 6.9, with 1 mM each of $MgSO_4$, EGTA and DTT, and either 1 mM GTP or 1 mM ATP. Purified tubulin was prepared from thrice cycled microtubule protein, including a MAP depleting step in 0.5 M Pipes and 10% DMSO followed by phosphocellulose chromatography. This protein eluted from the column at concentrations up to 50 mg/ml. Deviations from the standard assembly conditions are as described in the figure legends. The protein was stored in liquid nitrogen until use.

Time-resolved X-ray scattering

The X-ray experiments were performed on instrument X33 of the EMBL Outstation at the DESY synchrotron laboratory, Hamburg (Koch and Bordas, 1983), equipped with a rapid temperature jump cell (Renner *et al.*, 1983). The protein was filled into the chamber (depth 1 mm, covered with 50 μm mica windows on both sides) at 0−40°C, and the reaction was started by raising the temperature to 37°C within a few seconds. Scattering patterns were recorded in 2−6 s intervals on a position-sensitive detector integrating azimuthally over 90° (Bordas and Hendrix, unpublished) and analyzed as described (Mandelkow *et al.*, 1980; Bordas *et al.*, 1983; Spann *et al.*, 1987).

Electron microscopy

Assembly and oscillations were started by placing the cold sample in a water bath at 37°C. Aliquots were removed in 30-s intervals, diluted 20-fold into stabilizing buffer (assembly buffer plus 25% glycerol, for details see legend to Figure 2) to achieve a concentration suitable for electron imaging. Within a few seconds a drop was placed on a carbon coated, glow discharged grid and stained with 2% uranyl acetate. Images were recorded on a Philips CM12 electron microscope using Kodak Electron Image film.

Light scattering of oscillating microtubule samples

Observation by video imaging. Cold PC-tubulin was filled into a test tube (diameter 5 mm) and placed into a 37°C water bath at time 0. Images were recorded by a video camera connected to a Imaging Technologies image processing unit.

Observation in a Beckman DU 40 spectrophotometer by absorption at 350 nm. The sample holder and temperature jump device were the same as in the X-ray experiments (sample depth 1 mm). The formation of microtubules was ascertained in parallel X-ray experiments since light scattering does not discriminate between microtubules and oligomers.

Determination of protein-bound nucleotide by HPLC

50 μl aliquots of an oscillating solution were freed of unbound nucleotide by rapidly passing them through a Sephadex G25 column (1 ml insulin syringe). The eluted protein was precipitated by 2.5% perchloric acid and pelleted in a Biofuge (Heraeus). The supernatant was neutralized with KOH to precipitate the perchloric acid, spun in a Beckman TL100 ultracentrifuge (50 000 r.p.m.), 20 μl of the supernatant were injected into a Beckman M114 HPLC unit equipped with a variable wavelength UV detector (model 165), autoinjector (Abimed), and Shimadzu CR3A integrator. Absorption of nucleotides was monitored at 254 nm. The column was ODS-Ultrasphere (Altex), running buffer 50 mM phosphate pH 6.5, 1 mM tetrabutyl ammonium bromide, and 3% acetonitrile.

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