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Microtubule assembly and oscillations induced by flash photolysis of caged-GTP

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Abstract. Microtubule assembly and oscillations have been induced using the rapid liberation of GTP by UV flash photolysis of caged-GTP and monitored by time-resolved X-ray scattering. The flash photolysis method of achieving assembly conditions is much faster than the temperature jump method used earlier (msec vs. s range). However, the structural transitions and their rates are similar to those described previously. This means that the rates of the transitions in microtubule assembly observed before are determined by the protein itself, and not by the rate at which assembly conditions are induced. The advantages and limitations of using the photolysis of caged-GTP in microtubule assembly studies are compared with temperature jump methods. Caged-GTP itself reduces the rate of microtubule assembly and oscillations at m*M* concentrations, consistent with a weak interaction between the nucleotide analogue and the protein. X-rays are capable of slowly liberating GTP and other breakdown products from caged-GTP, even in the absence of UV flash photolysis, thus causing an apparent "X-ray-induced" microtubule assembly. This effect depends on the X-ray dose but is independent of the caged-GTP concentrations used here (m*M* range), suggesting that the breakdown of caged-GTP is caused not by the direct absorption of X-rays by the compound but by another intermediate reaction such as the generation of radicals by the X-rays.

Key words: Assembly – Caged-GTP – Microtubules – Oscillation – Synchrotron radiation – Tubulin – X-ray scattering

Introduction

Microtubule self-assembly can be observed in vitro in the presence of GTP at 37 °C. For studies of microtubule polymerization the solutions must be changed from an inactive state, where the protein is present in the form of α-β-heterodimers (the tubulin subunits) or small oligomers such as tubulin rings, to an active state, where the tubulin-GTP complex is capable of assembly. In order to observe the kinetics of the subreactions the shift to assembly conditions must be rapid compared to the structural transitions of the protein.

In previous studies by time-resolved X-ray scattering we achieved this by a rapid temperature jump from 4 °C to 37 °C (Mandelkow et al. 1980; Bordas et al. 1983; Spann et al. 1987). A common argument against T-jump experiments is that living cells do not change the temperature to control microtubule assembly. Another objection is concerned with the half-time of the temperature jump (in the range of several sec) which limits the time resolution of the experiment. Alternatively, polymerization can be started by addition of GTP to prewarmed, GTP-free tubulin solutions. Here, too, a possible objection is that rapid mixing of tubulin solutions by conventional stopped-flow techniques may affect the initial state because of the high shear stresses involved. This would alter the dynamics of microtubules.

Another way to produce a rapid rise in GTP concentration is the photochemical release of GTP in situ from an inactive precursor such as caged-GTP. This is a compound analogous to caged-ATP, which has been useful in studies of fast kinetics in different fields such as muscle contraction and ion-channel activation (Kaplan et al. 1978; McCray et al. 1980; for review see McCray and Trentham 1989). Caged compounds can be cleaved by a flash of UV-irradiation, resulting in the liberation of nucleotide within a few milliseconds, i.e. more than two orders of magnitude faster than the T-jump.

The published experiments with caged-ATP prompted us to examine whether UV-photolysis of caged-GTP can be used to start microtubule self-assembly, thus

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol-O,O′-bis (2-amino ethyl ether)-N,N,N′,N′-tetraacetic acid; GDP, guanosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; caged-GTP, P^3-1-(2-nitrophenyl) ethyl ester of GTP; HPLC, high performance liquid chromatography; Mt-protein, microtubule protein (=tubulin +MAPs); MAP(s), microtubule-associated protein(s); PC-tubulin, phosphocellulose-purified tubulin; PIPES, piperazine-1,4-bis(2-ethane sulfonic acid); UV, ultraviolet light

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avoiding the undesirable side effects of T-jump or rapid mixing. Here we show that flash photolysis can induce both microtubule assembly and synchronous oscillations. The main features of these reactions are similar to the ones observed previously. This means that – to a first approximation – it does not matter whether the conditions for assembly (37 °C, GTP) are reached within seconds or milliseconds. The use of caged-GTP in this type of application has certain limitations which will be discussed; in particular, the reduction of the rate of microtubule assembly by caged-GTP and the generation of GTP by X-rays (even without UV flash) must be taken into account.

Materials and methods

Microtubule protein and PC-tubulin were prepared from pig brain as described (Mandelkow et al. 1985). Protein solutions in 100 m*M* Na-PIPES (pH 6.9 at 20 °C), with 1 m*M* each of $MgSO_4$, EGTA and DTT were stored in liquid nitrogen until use. The microtubule assembly buffer was typically 100 m*M* PIPES, 1 m*M* $MgSO_4$, 1 m*M* EGTA, 5 m*M* DTT, and several m*M* GTP or caged-GTP. Oscillation buffers were similar to assembly buffers except for 20 m*M* $MgCl_2$ and 60 m*M* NaCl.

Caged-GTP was obtained from Calbiochem Corp. (La Jolla, CA). The purity was 99% as judged by HPLC. Solutions of 100 m*M* caged-GTP in 100 m*M* PIPES (pH 6.9) were prepared and stored at −70 °C before use.

Photolysis of caged-GTP was achieved by UV-irradiation using a xenon flash lamp with an ellipsoidal mirror as light collector (Scientific Instruments Dr. K. Güth, Heidelberg, FRG, model JML-87; see Rapp and Güth 1988). The electric discharge energy was up to 300 J per flash; the energy release as UV radiation at wavelength between 300 and 400 nm was about 0.5 J per flash at maximum discharge. The irradiated area was about 30 mm^2. In order to increase the yield of GTP, the transmitted light was reflected by a spherical mirror behind the sample. Liberation of GTP occurs within a few milliseconds (McCray et al. 1980). Flash photolysis of 10 m*M* caged-GTP solutions in capillaries resulted in 83% GTP yield at maximum flash intensity as judged by HPLC, but in the X-ray experiment it was only about 30% (see below).

The purity of caged-GTP and the extent of cleavage after irradiation were determined with a Beckman M114 HPLC unit equipped with a variable wavelength UV detector (model 165), a Shimadzu CR3A integrator, and a recorder for monitoring the UV spectra. A 150 × 4.6 column with Spherisorb ODS 5 µm packing (Bischoff Chromatography, Leonberg, FRG) was used. A linear gradient was applied over 10 min from buffer A (2 m*M* tetrabutyl ammonium bromide in 50 m*M* phosphate buffer, pH 6.5) to buffer B (40% acetonitrile, 60% buffer A). The flow rate was 1 ml/min. 20 µl aliquots of 100 µM nucleotide solutions were injected and the UV-absorption at 254 nm was monitored. The peaks of GTP and caged-GTP were identified by wavelength scans at the peak maxima.

The X-ray experiments were performed on instrument X33 of the EMBL Outstation at the DESY synchrotron laboratory, Hamburg (Koch and Bordas 1983; Boulin et al. 1986). For time-resolved X-ray scattering, tubulin solutions were placed in a 1 mm thick chamber covered with mica windows (same as the one used for rapid T-jump experiments, see Renner et al. 1983). Data collection, reduction, and interpretation were as described previously (Bordas et al. 1983; Spann et al. 1987). In time-resolved X-ray scattering experiments on flash induced tubulin assembly, the cell was mounted on a positioning device that allowed the cell to be moved under remote control to a position in front of the flash lamp and back to the X-ray beam within about one second. For these experiments, we used particularly thin mica windows of 5 to 10 µm thickness in order to reduce the UV-absorption. The absorption of 10 µm mica plates is about 20% at 350 nm and shows a sharp increase at shorter wavelengths. Because of the absorption loss, the amount of GTP liberation was only about 30% at maximum flash intensity. In addition, because of the strong absorption of UV light by caged-GTP, the liberation of GTP varies non-linearly across the depth of the chamber. Assuming an average absorption coefficient of 460 $M^{-1}\,cm^{-1}$ (corresponding to an effective wavelength of 350 nm), the intensity of the UV-beam decreases by a factor of about 0.6 per mm pathlength in a 5 m*M* caged-GTP solution. Back reflection of the transmitted light was used to increase the overall intensity and to reduce the spatial variation of irradiation.

Turbidity measurements were done in a Beckman DU 40 spectrophotometer by absorption at 400 nm. The sample holder and temperature jump device were the same as in the X-ray experiments.

For measurements of the liberation of GTP by X-rays, 1 to 3 µl of 10 m*M* caged-GTP in fused silica capillaries of 0.7 to 1.5 mm diameter with a 10 µm thick wall (Glas-Müller, Berlin) were placed into the X-ray beam for 25–125 min at room temperature. After irradiation the solution was diluted with 100 µl water and analyzed by HPLC. The X-ray intensity was monitored by two ionization chambers (parallel plate capacitor) before and behind the sample. Absolute intensities were deduced from the ionization currents using a value of 34 eV for the mean ionization energy in dry air. In calculating the number of photons absorbed by the solution, the intensity was assumed to be homogeneous across the beam. Corrections were made for absorption by the capillary and for evaporation of the solvent.

Results

(a) Microtubule assembly and oscillations induced by T-jump or photolysis of caged-GTP

Microtubules are assembled at 37 °C in the presence of GTP. The reaction usually takes the form of an approach to a new steady state (see below), but it can also appear as a series of oscillations where states of higher and lower degree of polymerization alternate with a periodicity in

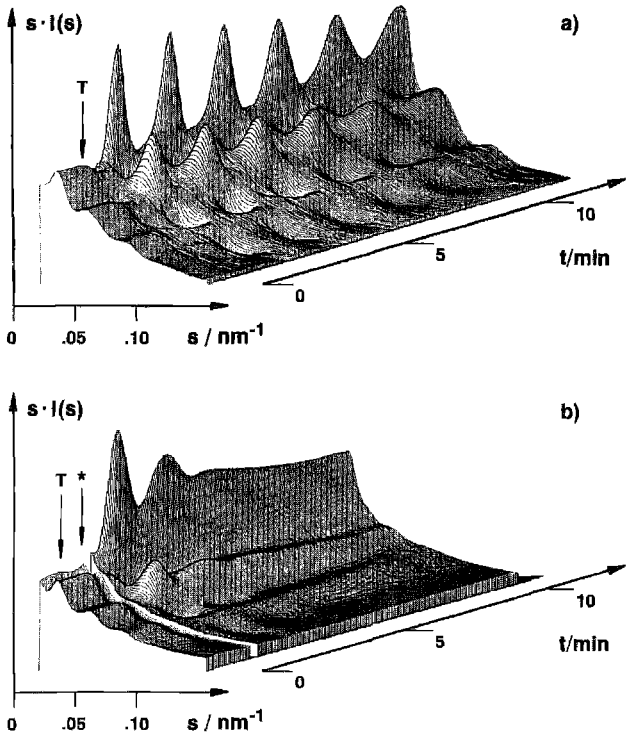

Fig. 1. Oscillations in microtubule assembly and disassembly induced by a temperature jump (**a**) and by flash photolysis of caged-GTP (**b**). Microtubule protein, 31 mg/ml, in 100 m*M* PIPES pH 6.9, 5 m*M* DTT, 1 m*M* EGTA, 55 m*M* NaCl, 18 m*M* $MgCl_2$ and 5 m*M* of GTP (**a**) or caged-GTP (**b**), respectively. **a** Initially, the protein solution with 5 m*M* GTP was at 4 °C; the side maxima of the scattering traces are typical of tubulin ring oligomers. The temperature was raised to 37 °C (arrow, half time about 8 s), leading first to a phase of ring dissolution, then to pronounced oscillations of microtubule assembly with a periodicity of about 1.8 min. The experiment was stopped after 12.8 min. **b** The solution with 5 m*M* caged-GTP was heated to 37 °C (arrow T) and after 30 s a single light flash of maximum intensity (300 J electric discharge energy) was given (arrow*), liberating about 1.5 m*M* GTP. This leads to oscillations similar to **a**, except that they are strongly damped. This can be explained by the lower concentration of GTP which reduces the efficiency of nucleation and causes a faster increase of the GDP/GTP ratio

the minute range (Pirollet et al. 1987; Carlier et al. 1987; Mandelkow et al. 1988; Lange et al. 1988). Since assembly depends on the hydrolysis of GTP (one for every tubulin dimer incorporated into a microtubule) the oscillations are rather sensitive to the amount of GTP present in the solution.

In Fig. 1 we compare oscillations of microtubule protein induced by temperature jump (Fig. 1 a) and flash photolysis (Fig. 1 b). The projection plots show the evolution of the X-ray scattering curves with time. The interpretation has been described previously (Mandelkow et al. 1988; Lange et al. 1988). In both cases the initial solution contains tubulin subunits and oligomers (mainly rings of 35 nm diameter), as judged from the low "central scatter" and the positions of the side maxima ($S=$ 0.035 nm^{-1} and 0.065 nm^{-1}). When microtubules are formed, the central scatter rises steeply in both experiments (equivalent to an increase in the degree of polymerization), and new side maxima appear around 0.05 and 0.095 nm^{-1} (corresponding to the microtubule diameter of about 25 nm).

In Fig. 1 a the initial solution (containing 5 m*M* GTP) was at 4 °C, the temperature was shifted to 37 °C (arrow T) and kept constant thereafter. After the T-jump the intensity briefly decays because ring oligomers are destabilized, but then the solution oscillates between a higher and lower state of microtubule assembly with a periodicity of about 1.8 min. Six cycles can be observed before the experiment was terminated; the oscillations are gradually damped concomitant with the consumption of GTP and the buildup of GDP, an inhibitor of microtubule assembly.

In Fig. 1 b, the solution containing 5 m*M* caged-GTP was raised to 37 °C (arrow T) before GTP was liberated by single flash (arrow*). There is a slight increase in central scatter starting even before the flash. This could be due to slow non-specific aggregation, a common feature with tubulin kept at 37 °C, but other effects play a role as well (the slow X-ray induced liberation of GTP, see below). However, the main feature of the experiment is that there are microtubule oscillations similar to Fig. 1 a, except that they are much more strongly damped so that only two cycles are clearly visible. This is simply explained by the lower concentration of GTP; a single flash liberates only about 30%, equivalent to 1.5 m*M* GTP. At these lower GTP concentrations the nucleation of microtubules is less efficient, leading to a smaller microtubule number concentration, and the GDP/GTP ratio becomes quickly inhibitory. Both effects contribute to the reduction of oscillation cycles (see Lange et al. 1988; Obermann et al. 1990), although other side effects due to caged-GTP itself must also be considered (see below).

We now turn to the assembly of PC-tubulin in standard conditions (i.e. buffer conditions supporting assembly but not oscillations, see Methods) and investigate the relationship between a GTP-jump and a T-jump. The curves in Fig. 2 show the time dependence of the "central scatter" between 0.015 and 0.020 nm^{-1} which are representative of the weight average degree of polymerization. Figure 2 a is a control experiment in which assembly and disassembly is induced by changing the temperature, as described earlier (Mandelkow et al. 1980). After the first T-jump from 4 to 37 °C there is a small undershoot, caused by oligomer dissolution, followed by microtubule assembly, and then disassembly induced by the reverse T-jump. Since tubulin is at 0.35 m*M* and GTP at 2 m*M*, the GTP suffices for several cycles of which only two are shown, although assembly becomes successively slower because of the buildup of GDP.

Figure 2 b is another T-jump experiment, except that the solution contained initially 3 m*M* caged-GTP and was irradiated by a single flash (arrow*) 3 min before the T-jump, liberating close to 1 m*M* GTP. There is no detectable change in the scattering pattern after the flash, showing that the photolysis of caged-GTP has no major effect on the structures at low temperature. After the T-jump the protein polymerizes with the same general characteristics as in Fig. 2 a, except that assembly is slower than in the control. The undershoot develops with nearly the same rate as in Fig. 2 a since the temperature induced

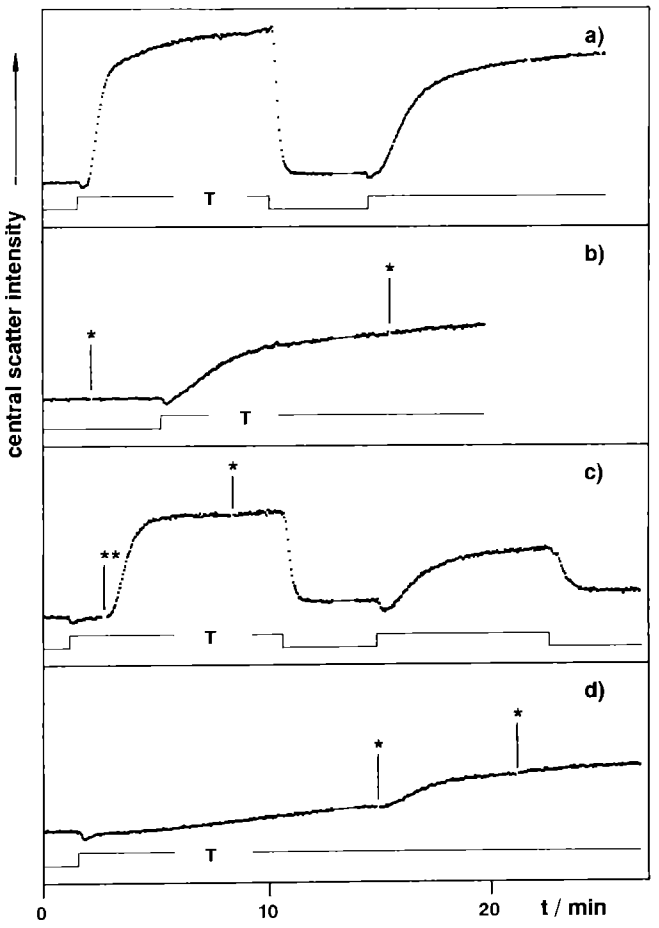

Fig. 2a–d. Time-resolved X-ray scattering from tubulin solutions, showing microtubule polymerization induced by temperature jumps (**a, b**) and GTP-concentration jumps (**c, d**). The solutions were prepared with 2 m*M* GTP (**a**) or 3 m*M* caged-GTP (**b, c, d**). In experiment b, caged-GTP was photolyzed to GTP prior to the temperature jump (one flash at the time indicated by *). In the GTP-concentration jump experiments **c** and **d**, polymerization was started by caged-GTP photolysis, with the solution held at 37 °C for 1.5 min (**c**, two flashes) and for 13 min (**d**, one flash), respectively. Solutions contained 35 mg/ml PC-tubulin, 100 m*M* PIPES, 1 m*M* $MgSO_4$, 1 m*M* EGTA, and 5 m*M* DTT

dissolution of oligomers does not depend on GTP (see Spann et al. 1987).

We now reverse the order of T-jump and flash (Fig. 2c). After the T-jump we observe the undershoot, but assembly does not set in until GTP is released (two flashes, equivalent to about 1.5 m*M* GTP, 1.5 min after the T-jump). Immediately after the flashes the scattering patterns are the same as before, allowing us to make two conclusions. One is that the generation of GTP has no influence on the structures in solution per se, even at 37 °C (compare Fig. 2b). Secondly, microtubule assembly remains intrinsically slow, even when assembly conditions are induced in milliseconds (by flash photolysis) rather than seconds (as with T-jumps). Microtubule assembly sets in after 15 s, with kinetics similar to Fig. 2a and faster than Fig. 2b, consistent with the higher concentration of GTP.

Finally, we repeat the above experiment, except that the time between the T-jump and the release of GTP is increased to 13 min (Fig. 2d). Following the T-jump there is the usual undershoot, but now one also observes a slow increase in the scattering intensity. This is not due to microtubule formation (as judged from the pattern at higher angles, not shown), but rather to an unspecific aggregation and denaturation of the protein at 37 °C. When a single flash is given 13 min after the T-jump, only part of the protein (about 20%) slowly assembles, and a second flash at $t = 21$ min has no further effect.

The main conclusions from the experiments described so far are the following: In our earlier studies we had employed T-jumps with half times of several seconds to induce assembly. Because dissolution of cold-stabilized oligomers occurs with a rate comparable to that of the T-jump, one could argue that this is rate limiting, thus obscuring some intrinsic rate of the assembly reaction itself. Moreover, we could have overlooked fast subreactions. In our GTP-concentration jump experiments oligomer dissolution and microtubule assembly are clearly separated, showing that the observed rates of microtubule assembly (both in flash photolysis and T-jump experiments) are intrinsic to the protein.

Similar conclusions can be drawn from another set of experiments analogous to those of Fig. 2, but in buffer conditions favoring oscillations, and in the presence of 3 m*M* caged-GTP (Fig. 3). In Fig. 3a there is first a T-jump to 37 °C, followed by a single flash. After the flash there is first a pronounced lag, typical of oscillatory conditions (about 1.5 min), followed by a single assembly and disassembly. In Fig. 3b the T-jump is preceded by four flashes, resulting in two strongly damped cycles. In Fig. 3c the order is reversed, i.e. the T-jump is followed by four flashes, leading to similar oscillations as in Fig. 3b. Thus it appears that the order of flash and T-jump does not matter as long as they are done within a short time. Finally, when the protein is kept at 37 °C for an extended period without GTP it tends to aggregate and lose its competence for assembly and oscillations (not shown, similar to Fig. 2d).

(b) Effect of caged-GTP on GTP-dependent microtubule oscillations

Microtubule oscillations require fairly high protein concentrations, say from 100 μM up; for observing extended oscillations one needs a several-fold excess of GTP over tubulin, and because of the low efficiency of flash photolysis the concentration of caged-GTP must be even higher, on the order of several m*M*. At these concentrations it is pertinent to ask if caged-GTP itself has an influence on the assembly behavior. The light scattering experiment of Fig. 4 shows that this is indeed the case. Oscillations of a solution of 260 μM PC-tubulin were induced by T-jump in the presence of a constant amount of 4 m*M* GTP and additional 0–10 m*M* caged-GTP, without flash photolysis. Similar experiments were done with varying Mg^{++} concentrations to exclude a trivial Mg^{++}-depletion effect by the increasing amount of nucleotide. It is clear that caged-GTP itself is an inhibitor of oscillations. It reduces both the initial assembly rate, the

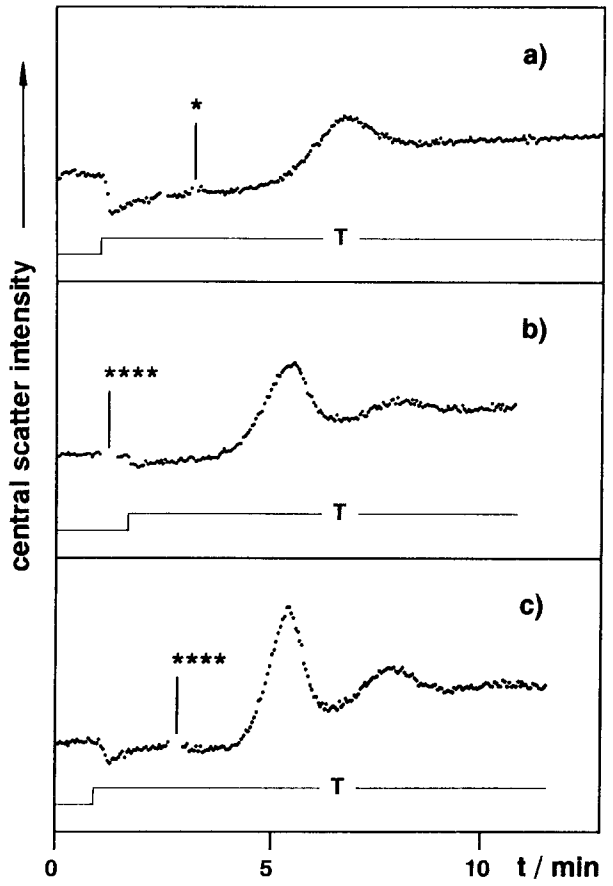

Fig. 3a–c. Time-resolved X-ray scattering from tubulin solutions in oscillation buffer prepared with 3 m*M* caged-GTP (35 mg/ml PC-tubulin in 100 m*M* PIPES, 1 m*M* $MgSO_4$, 1 m*M* EGTA, 5 m*M* DTT, 20 m*M* $MgCl_2$, 60 m*M* NaCl). In experiment **a**, oscillation was started by a single UV-flash at 37 °C (2 min after T-jump to 37 °C), in each of the other experiments, a sequence of 4 flashes was used. Photolysis of caged-GTP was performed 2 min after T-jump in **a**, 0.5 min before T-jump in **b**, and 1.5 min after T-jump in **c**

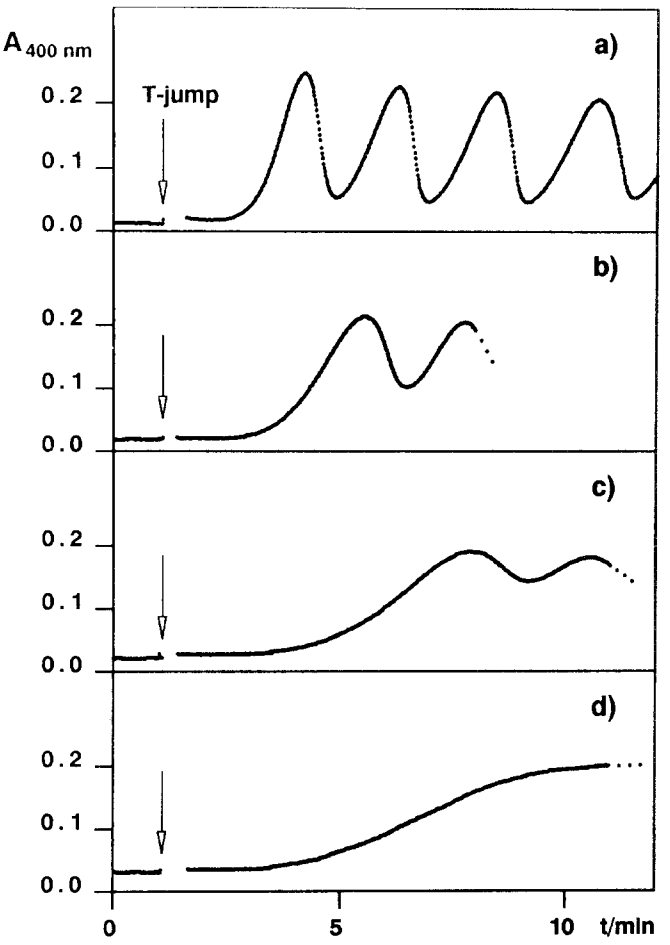

Fig. 4a–d. Time-dependence of the turbidity ($A_{400\ nm}$) of tubulin solutions in oscillation buffer with 4 m*M* GTP, showing the effect of additional caged-GTP: **a** no caged-GTP present (control), **b** 2 m*M* caged-GTP, **c** 4 m*M* caged-GTP, and **d** 10 m*M* caged-GTP present. The solutions contained 26 mg/ml PC-tubulin in 100 m*M* PIPES, 20 m*M* $MgCl_2$, 60 m*M* NaCl, and 1 m*M* DTT. In this series of experiments the lag time preceding assembly increases from 1.4 to 2.4 min, the maximum turbidity (after subtracting the initial turbidity) decreases from 0.23 to 0.17, the amplitude decreases from 84% to 0% of the maximum, and the frequency of the oscillations decreases from 0.48 to 0 min^{-1}

amplitude, the maximum degree of assembly, and the frequency. For flash photolysis experiments this means that the dominant effects (i.e. microtubule assembly and oscillations) are due to the liberated GTP because the protein is inactive without it, but the remaining caged-GTP cannot be disregarded. Our general experience is that flash photolysis is less efficient than T-jump in promoting microtubule assembly or oscillations. In view of the data of Fig. 4 this is explained both by the low efficiency of GTP liberation and by the inhibitory effect of caged-GTP.

(c) X-ray induced assembly of microtubules in the presence of caged-GTP

Microtubule assembly requires GTP which in turn is released from caged-GTP by irradiation at UV wavelengths in the range of 300 to 350 nm (see McCray and Trentham 1989). Thus, in the absence of UV light flashes one would expect no microtubule formation. However, microtubule protein solutions did not behave according to the expectation and showed clear microtubule assembly even without UV flashes, albeit at a slow rate (as in Fig. 5a). We initially suspected that the caged-GTP was contaminated with GTP, but the HPLC assay showed that GTP was below detectability. At the same time we also noted that the assembly of microtubules was somehow related to the exposure to X-rays. The following experiments were designed to test if X-rays are capable of releasing GTP from caged-GTP.

In Fig. 5b the X-ray beam was shut off for 2 min during the experiment while the solution was kept at 37 °C. After the break the central scatter was slightly increased, but there was no sign of microtubules. After one minute of X-ray exposure microtubule formation started. This is similar to Fig. 5a, except that the onset of assembly is delayed by about 2 min. Figure 5c is a similar experiment where the X-ray beam was shut off for even longer (almost 3 min), resulting in a further delay of the onset of microtubule assembly. Finally, in Fig. 5d continuous irradia-

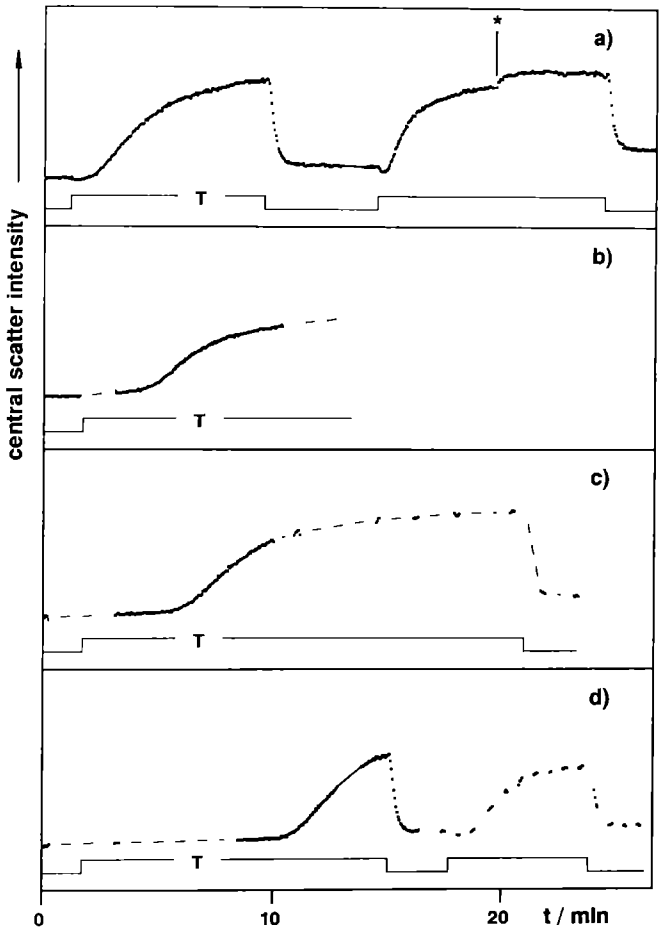

Fig. 5a–d. X-ray induced microtubule assembly in solutions of microtubule protein with 2 m*M* caged-GTP. The curves show the intensity of the central scatter; breaks in the curves occur when the X-ray beam was shut off. The temperature (T) of the specimen is schematically depicted by thin lines (low, 4 °C, high, 37 °C). Arbitrary scale, same in all traces. About 80% of the protein polymerizes compared with the control using GTP. Solutions contained 39.5 mg/ml of microtubule protein in 100 m*M* PIPES pH 6.9, 0.7 m*M* $MgSO_4$, 0.7 m*M* EGTA, and 5 m*M* DTT. **a** The sample was irradiated by X-rays for about 1 min before data collection was started. Slow microtubule assembly sets in more than 2 min after the start of irradiation. After depolymerization of the microtubules by cooling down to 4 °C, microtubule formation could be started once more by raising the temperature to 37 °C. This time, polymerization was even faster than the first time. This may be caused by a higher number concentration of nuclei at the beginning of the second polymerization, and/or by accumulation of GTP during X-ray irradiation at low temperature (biphasic polymerization kinetics). The maximum extent of polymerization was reached after UV-photolysis (*, one flash). **b** The sample was irradiated at 4 °C for 2 min, then the X-ray was shut off for 2 min (break in data collection) and the temperature was raised to 37 °C. Microtubule assembly sets in at about 3 min total irradiation time. **c** At 4 °C the sample was irradiated during a short period of about 10 s in order to record the initial scattering pattern. Continuous X-ray irradiation and data collection was started 1.5 min after the T-jump. Microtubule formation sets in after about 3 min of irradiation. During the assembly the X-ray beam was shut off except for short sampling periods, but assembly continued without interruption. **d** The experiment was started at 4 °C, the T-jump was done at $t = 1.5$ min. During the first 8 min, the X-ray beam was shut off, except for two brief 15 s exposures to ascertain that the X-ray pattern remained constant during that time. Continuous irradiation was started 6.5 min after the T-jump. Microtubule assembly sets in after about 3 min of irradiation and data collection did not start until 6.5 min after the T-jump; until then only brief test exposures were taken. The experiment shows that the pattern remains essentially constant until 3 min after the start of continuous irradiation. These data suggest that the generation of the GTP that causes the assembly depends on the irradiation by X-rays. Assembly sets in after a lag time of about 3 min; however, once started it cannot be interrupted by shutting off the X-ray beam.

As controls we note the following observations: After X-ray induced microtubule assembly we moved the sample chamber to another position which was not irradiated previously; it did not contain microtubules, but they formed after a lag period. This means that the GTP is generated within the irradiated volume, with very little diffusion outside it (consistent with the calculated diffusion rates, see Discussion). Secondly, without caged-GTP there is no assembly, showing that caged-GTP is a prerequisite. The lag before assembly depends on the effective exposure time during which a critical X-ray dose is accumulated, but it does not show a significant dependence on the concentrations of caged-GTP in our concentration range (up to 10 m*M*, not shown). This is in contrast to the cleavage of caged-GTP by UV light; the implications for the mechanism of GTP liberation will be discussed later.

(d) Cleavage of caged-GTP by X-ray irradiation

To quantify the rate of GTP liberation by X-rays, we irradiated 10 m*M* caged-GTP solutions with defined X-ray doses and determined the concentrations of GTP and caged-GTP by HPLC. Irradiation resulted in the appearance of at least 9 new peaks. The strongest one is due to GTP as judged by its retention time and absorption spectrum. Other pronounced peaks are due to GDP and GMP. The measurements are compatible with a linear relationship between the number of absorbed photons and the number of caged-GTP molecules consumed by X-ray irradiation (Fig. 6, open circles). About one third of the decomposed caged-GTP molecules are found as GTP (Fig. 6, lower trace). The apparent efficiency of GTP production is about 7 molecules per photon (at 8.3 keV photon energy). Since GTP is also decomposed during irradiation (roughly at the same rate as caged-GTP, data not shown) the actual efficiency of GTP production is about 10% higher.

With this information it is possible to calculate the concentration of GTP which has been liberated by X-ray irradiation at the time when polymerization becomes evident in the experiments of Fig. 5, assuming that GTP liberation in protein solutions is comparable to that in pure caged-GTP solutions. This concentration amounts to about 30 μM (10 μM per min, see Discussion). Considering that the protein contains initially bound GDP (about 400 μM in Fig. 5), and that GTP and GDP have similar binding constants, about 10% of the dimers would have bound GTP when polymerization sets in. This is about 10 times the critical concentration of microtubule protein (about 1–3 μM) and explains why the

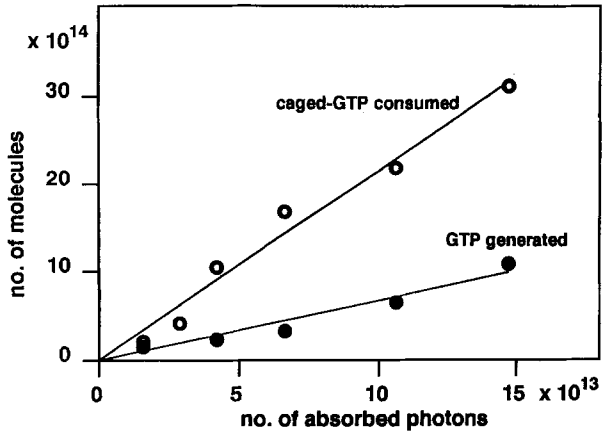

Fig. 6. Dose dependence of X-ray induced cleavage of caged-GTP (open circles) and generation of GTP (filled circles). Up to 3 µl of 10 m*M* caged-GTP solutions were irradiated at room temperature for periods ranging from 25 min to 125 min. One absorbed photon generates about 7 molecules of GTP

protein becomes assembly-competent. By contrast, PC-tubulin has a critical concentration of 30 µM or more, so that a much longer exposure to X-rays would be required before assembly would set in (for example, in Fig. 2d no microtubules are observed even after 15 min exposure).

Once polymerization has started, incorporation of tubulin dimer into microtubule procedes with a rate constant that is at least one order of magnitude greater than the rate of GTP liberation. This suggests that the slow polymerization observed after short periods of X-ray irradiation (Fig. 5) is largely due to the addition of GDP · tubulin (compare Bayley and Manser 1985; Hamel et al. 1986).

Discussion

(a) Intermediate steps in tubulin assembly

In previous X-ray studies we had employed a rapid T-jump in order to initiate microtubule assembly (Mandelkow et al. 1980; Bordas et al. 1983; Spann et al. 1987) or oscillations (Mandelkow et al. 1988; Lange et al. 1988). We broadly distinguished between pre-nucleation events, nucleation, elongation, and post-assembly events. The pre-nucleation events consist mainly of a temperature-dependent dissolution of tubulin oligomers, suggesting that oligomers are not involved in microtubule nucleation or elongation. Post-assembly events can include several types of reactions, such as a decay to a lower degree of assembly after a transient assembly maximum (termed overshoot) or a series of oscillations (as in Fig. 1). Both of these can be related to the dynamic instability of microtubules (see Mitchison and Kirschner 1984). Superimposed on this is the slow but noticeable denaturation and aggregation of tubulin at 37 °C which is further aggravated by the exposure to X-rays.

Most of these reactions are slow compared to the T-jump, except for the pre-nucleation events which have a comparable rate (Spann et al. 1987). If there were much faster initial reactions they would possibly be overlooked in T-jump experiments. The present results essentially confirm the conclusions derived from the T-jump experiments. Microtubule assembly and oscillations take place at similar rates as observed previously, i.e. the kinetics are determined by the protein interactions and not by the method of initiating assembly. The equilibrium between oligomers and subunits is temperature-dependent but does not require GTP. Thus an undershoot is seen in a T-jump experiment whether GTP is present or not; when a UV flash is applied at 37 °C there is no additional undershoot, at least in the conditions studied here. Thus the observed pre-nucleation events result from the temperature shift (i.e. oligomers are more stable at 4 °C than at 37 °C) but are not a prerequisite for microtubule assembly. This is consistent with the idea that the oligomerization of tubulin must be regarded as independent of microtubule assembly, and, in particular, that the observed oligomers are not involved in microtubule nucleation.

(b) Comparison of T-jump and flash photolysis for inducing microtubule assembly

For studying microtubule assembly in vitro using X-ray scattering one has to satisfy several constraints: The main advantage of X-rays is that the scattering pattern contains more structural information than light scattering. However, because of the low scattering cross-section of X-rays and its rapid decay at increasing scattering angles one has to use fairly high protein concentrations, on the order of 100 µM and more in our case. For observing oscillations one also needs these high concentrations, independently of the method of observation. Since GTP is rapidly consumed, especially during oscillation, and since GDP is a strong inhibitor of assembly, one needs a large initial excess of GTP (on the order of m*M*) and, consequently, an even larger concentration of caged-GTP.

The major advantage of the flash photolysis method is its very good time resolution since GTP is generated within a few milliseconds. Thus, if the reactions under study took place on a sub-second scale, flash photolysis would be the method of choice. In the present case it turns out that microtubule nucleation and assembly are slower than the T-jump, so that even with flash photolysis no new types of subreaction can be detected. This is one major result of this study, but once it has been established the disadvantages of the flash photolysis method tend to outweigh its advantages, for the following reasons: *(a)* The concentration of the liberated GTP is not homogeneous along the depth of the chamber because of the strong absorption of UV light by caged-GTP; it is highest in the front and decays towards the back of the chamber. The problem can be reduced but not eliminated by a back-reflecting mirror. *(b)* Flash photolysis of caged-GTP liberates not only GTP but also 2-nitrosoacetophenone which has deleterious effects on biological materials (Walker et al. 1988). Its effect on microtubule assembly is unknown but it could result in the oxidation of SH groups on tubulin, some of which are known to be important for microtubule assembly (Mellon and Rebhun

1976). This effect would add to that of caged-GTP alone which also inhibits microtubule assembly, even without a UV flash (see below and Fig. 4). At any rate, the inhibition could not be reduced significantly by addition of DTT (a radical scavenger) in m*M* concentrations. *(c)* X-rays are capable of liberating GTP from caged-GTP and thus complicate experiments where low concentrations of GTP matter, as discussed later.

(c) Cleavage of caged-GTP and generation of GTP by X-rays

The number of GTP molecules generated from caged-GTP by X-ray absorption is proportional to the X-ray dose (Fig. 6), but in contrast to UV photolysis, it seems not to be proportional to the concentration of caged-GTP in the range used here (2–10 m*M*). This could be explained by assuming that in both cases the number of GTP molecules created is proportional to the number of absorbed photons. While UV absorption (photon energy 3–4 eV) is due to the caged-GTP molecules themselves, X-ray photons (8.3 keV) are absorbed mainly by the abundant water molecules. Thus, the observed X-ray generation of GTP seems to be an indirect effect: The X-ray photons produce free radicals which in turn could be responsible for the breakdown of caged-GTP. If the second reaction is rapid and not limited by the availability of caged-GTP, the generation of GTP would depend on the number of absorbed photons only. As an example, let us assume a typical experiment with an irradiated volume of 5 µl, a concentration of 2 m*M* caged-GTP, and $5 \cdot 10^{12}$ X-ray photons absorbed per minute. Every absorbed photon produces a large number of radicals (≈ 500, Spinks and Woods 1976) which in turn destroy 20 molecules of caged-GTP in total, 7 of them being converted to GTP (Fig. 6). This leads to an increase of the GTP concentration in the irradiated volume of about 10 µM per minute. Here it is assumed that the diffusion of GTP out of the irradiated volume is negligible (with a diffusion constant of about $5 \cdot 10^{-6}$ cm^2 s^{-1}, the mean distance traveled by a diffusing GTP molecule in 20 min is about 1 mm, according to $\langle x^2 \rangle = 2$ Dt).

Once the amount of GTP generated by X-rays has reached some threshold value microtubule assembly sets in. In the case of microtubule protein (Fig. 5) this value was about 30 µM, roughly ten times the critical concentration (1–3 µM tubulin). This is reasonable considering that there is an initial level of GDP equimolar to the protein concentration, about 400 µM in Fig. 5, which is an inhibitor of assembly. In the case of PC-tubulin the critical protein concentration is much higher, about 30 µM and above. Thus, to induce assembly by X-ray liberation of GTP would require much longer exposure times which explains why no microtubule formation is observed during the initial 15 min in Fig. 2d.

In summary, it appears that the production of GTP by X-rays cannot be neglected, especially in cases where the protein under investigation binds GTP strongly so that even small amounts influence its behavior, as is the case here. Unlike with T-jump experiments, care must be taken to keep the irradiation of the specimen prior to the UV flash at a minimum. Secondly, the liberation of GTP is probably an indirect effect that is mediated by radicals but not by the direct absorption of X-rays by caged-GTP.

(d) Interaction between caged-GTP and tubulin

The rationale for using caged-GTP rests on the assumption that this compound does not interfere with the reactions to be observed. This is not the case here, as illustrated in Fig. 4. In this context we note that several authors have observed a general nucleotide effect on microtubule assembly consistent with a second weak binding site (Jameson and Caplow 1980; Zabrecky and Cole 1982; O'Brien and Erickson 1989). We also note that another GTP binding protein, ras p21, binds caged-GTP as well, with a 100-fold lower affinity than GDP (Schlichting et al. 1989). Whether caged-GTP binds to the exchangeable GTP binding site of tubulin (E-site) with high affinity comparable to that of GTP (K_d below 0.1 µM, Correia et al. 1987) or to a different binding site with K_d in the m*M* range cannot be decided from the inhibition experiment of Fig. 4. However, the fact that microtubule assembly sets in when only a small fraction of caged-GTP is converted to GTP by X-rays suggests that binding to the E-site, if possible, is rather weak.

Finally we note that the rate and extent of polymerization found in X-ray induced microtubule assembly (Fig. 5) clearly exceeds that of GTP liberation. This implies that part of the tubulin molecules are incorporated into microtubules without bound GTP. In view of the findings of several other authors this seems not to be too surprising: Firstly, it has been shown that nucleotide-depleted tubulin or tubulin with bound GDP can assemble to a limited extent (e.g. Bayley and Manser 1985; Hamel et al. 1986; Lin and Hamel 1987). It seems possible that tubulin with caged-GTP bound to the E-site, if present, could also contribute to elongation. Secondly, it has been proposed that occupancy of a hypothetical second nucleotide binding site by caged-GTP could modify the assembly behavior (see Jameson and Caplow 1980; O'Brien and Erickson 1989). Thirdly, some nucleotide analogues can bind to the E-site with low affinity and stimulate tubulin polymerization synergistically at low concentrations (Hamel and Lin 1990; Mejillano et al. 1990). In conclusion, the X-ray induced assembly we observed seems consistent with these findings indicating that nucleation is not possible if there is no GTP present, but requirements on nucleotides for microtubule elongation are much less severe, especially in case of the high tubulin concentrations used here.

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References

Bayley PM, Manser EJ (1985) Assembly of microtubules from nucleotide-depleted tubulin. Nature 318:683–685

Bordas J, Mandelkow E-M, Mandelkow E (1983) Stages of tubulin assembly and disassembly studied by time-resolved synchrotron X-ray scattering. J Mol Biol 164:89–135

Boulin C, Kempf R, Koch M, McLaughlin S (1986) Data appraisal, evaluation and display for synchrotron radiation experiments: Hardware and software. Nucl Instrum Methods A249:399–407

Carlier MF, Melki R, Pantaloni D, Hill TL, Chen Y (1987) Synchronous oscillations in microtubule polymerization. Proc Natl Acad Sci USA 84:5257–5261

Correia JJ, Baty LT, Williams RC (1987) Mg^{++} dependence of guanine nucleotide binding to tubulin. J Biol Chem 262:17278–17284

Hamel E, Lin CM (1990) Reexamination of the role of nonhydrolyzable guanosine 5′-triphosphate analogs in tubulin polymerization: Reaction conditions are a critical factor for effective interactions at the exchangeable nucleotide site. Biochemistry 29:2720–2729

Hamel E, Batra J, Lin C (1986) Direct incorporation of guanosine 5′-diphosphate into microtubules without guanosine 5′-triphosphate hydrolysis. Biochemistry 25:7054–7062

Jameson L, Caplow M (1980) Effect of guanosine diphosphate on microtubule assembly and stability. J Biol Chem 255:2284–2292

Kaplan JH, Forbush B, Hoffman JF (1978) Rapid photolytic release of adenosine 5′-triphosphate from a protected analogue: Utilization by the Na:K pump of human red blood cell ghosts. Biochemistry 17:1929–1935

Koch MHJ, Bordas J (1983) X-ray diffraction and scattering on disordered systems using synchrotron radiation. Nucl Instrum Methods 208:461–469

Lange G, Mandelkow E-M, Jagla A, Mandelkow E (1988) Tubulin oligomers and microtubule oscillations: Antagonistic role of microtubule stabilizers and destabilizers. Eur J Biochem 178:61–69

Lin CM, Hamel E (1987) Interrelationships of tubulin-GDP and tubulin-GTP in microtubule assembly. Biochemistry 26:7173–7182

Mandelkow E-M, Harmsen A, Mandelkow E, Bordas J (1980) X-ray kinetic studies of microtubule assembly using synchrotron radiation. Nature 287:595–599

Mandelkow E-M, Herrmann M, Rühl U (1985) Tubulin domains probed by subunit-specific antibodies and limited proteolysis. J Mol Biol 185:311–327

Mandelkow E-M, Lange G, Jagla A, Spann U, Mandelkow E (1988) Dynamics of the microtubule oscillator: role of nucleotides and tubulin-MAP interactions. EMBO J 7:357–365

McCray J, Trentham D (1989) Properties and uses of photoreactive caged compounds. Ann Rev Biophys Biophys Chem 18:239–270

McCray J, Herbette L, Kihara T, Trentham D (1980) A new approach to time-resolved studies of ATP-requiring biological systems: Laser flash photolysis of caged ATP. Proc Natl Acad Sci USA 77:7237–7241

Mejillano MR, Barton JS, Nath JP, Himes RH (1990) GTP analogs interact with the tubulin exchangeable site during assembly and upon binding. Biochemistry 29:1208–1216

Mellon MG, Rebhun LI (1976) Sulfhydryls and the in vitro polymerization of microtubules. J Cell Biol 70:226–238

Mitchison T, Kirschner M (1984) Dynamic instability of microtubule growth. Nature 312:237–242

Obermann H, Mandelkow E-M, Lange G, Mandelkow E (1990) Microtubule oscillations: Role of nucleation and microtubule number concentration. J Biol Chem 265:4382–4388

O'Brien ET, Erickson HP (1989) Assembly of pure tubulin in the absence of free GTP: Effect of magnesium, glycerol, ATP, and nonhydrolyzable GTP analogs. Biochemistry 28:1413–1422

Pirollet F, Job D, Margolis RL, Garel JR (1987) An oscillatory mode for microtubule assembly. EMBO J 6:3247–3252

Rapp G, Güth K (1988) A low cost intensity flash device for photolysis experiments. Eur J Physiol 411:200–203

Renner W, Mandelkow E-M, Mandelkow E, Bordas J (1983) Self-assembly of microtubule protein studied by time-resolved X-ray scattering using temperature jump and stopped flow. Nucl Instrum Methods 208:535–540

Schlichting I, Rapp G, John J, Wittinghofer A, Pai E, Goody R (1989) Biochemical and crystallographic characterization of a complex of c-Ha-ras p21 and caged GTP with flash photolysis. Proc Natl Acad Sci USA 86:7687–7690

Spann U, Renner W, Mandelkow E-M, Bordas J, Mandelkow E (1987) Tubulin oligomers and microtubule assembly studied by time-resolved X-ray scattering: Separation of prenucleation and nucleation events. Biochemistry 26:1123–1132

Spinks JWT, Woods RJ (1976) An introduction to radiation chemistry. Wiley, New York

Walker J, Reid G, McCray J, Trentham D (1988) Photolabile 1-(2-nitrophenyl)ethyl phosphate esters of adenine nucleotide analogues. Synthesis and mechanism of photolysis. J Am Chem Soc 110:7170–7177

Zabrecky JR, Cole RD (1982) Effect of ATP on the kinetics of microtubule assembly. J Biol Chem 257:4633–4638