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Kinetics of the spontaneous organization of microtubules in solution

M. Somers and Y. Engelborghs

Laboratory of Chemical and Biological Dynamics, Katholieke Universiteit te Leuven, Celestijnenlaan 200 D, B-3030 Leuven, Belgium

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Abstract. Optically anisotropic zones occur spontaneously in solutions of microtubules. These tactoids, in which microtubules are arranged in parallel arrays, can be visualized by their birefringence. With microtubules assembled in the presence of associated proteins (MAPs), birefringence appears immediately after nucleation of polymerization, even at relatively low protein concentrations. It is not dependent on whether the assembly is initiated by temperature jump or by isothermal addition of GTP. With pure tubulin, assembled in buffers containing 25% glycerol or 4% dimethylsulfoxide and/or taxol, birefringence appears within a few hours, but it can be speeded up by gentle agitation. With tubulin assembled in the presence of MAPs, spontaneous orientation occurs simultaneously with polymerization. This may be due to the existence of more pronounced repulsive forces between microtubules when they are covered with MAPs. A simple calculation of the covolume, suggests that tactoid formation is expected for microtubules of lengths of 5 to 10 μm at protein concentrations in the range 1 to 3 mg/ml (as observed), and that repulsive forces will promote tactoid formation at even lower protein concentrations.

Key words: Tubulin – Microtubule – Alignment – Nematic liquids

Introduction

In eukaryotic cells, microtubules always appear to be organized. During mitosis the spindle structure is formed, in which all the microtubules originate from the centrosomes, with the (+) end being distal (Mitchison et al. 1986). Parallel bundles are found in the neighbourhood of the equatorial plane, where certain microtubules interact with the chromosomes at the kinetochores, others interact with the antiparallel microtubules which originated at the other pole. Even when the cells are not dividing, microtubules form a three dimensional network, mainly oriented from the nucleus towards the periphery (Osborn and Weber 1976). When taxol is added, the normal organization disappears, apparently because taxol lowers the critical concentration below the nucleating power of the centrosomes. New taxol-microtubules are formed which organize themselves into bundles (De Brabander et al. 1981; Turner and Margolis 1984). Bundles of microtubules are also formed in the presence of the enzyme glyceraldehyde-3-phosphate dehydrogenase (Kumagai and Sakai 1983; Huitorel and Pantaloni 1985). In the former cases, organization is largely due to the geometry of the nucleation centres. In the latter two cases, bundling is due to the existence of lateral attractive forces between microtubules.

Even in the absence of organizing centres or attractive forces, long linear molecules in solution are known to organize themselves spontaneously into zones of parallel arrays, so-called tactoids (Oster 1950; Briehl and Herzfeld 1979). Nematic liquids are formed in this way. The driving force for such spontaneous organization is the decrease in the mutually excluded volume when polymers diffuse from the perpendicular configuration into the parallel one (Onsager 1949). Given sufficient time, phase separations can occur because the density in the organized zones is slightly higher than in the rest of the solution. Such phase separations have been observed by Oster (1950) in the case of tobacco mozaic virus. Phase separations, as well as macroscopic anisotropic zones, can be visualized by their birefringence.

Microtubules show a similar behaviour (Somers and Engelborghs 1987; Hitt et al. 1987). Upon the polymerization of tubulin, macroscopic anisotropic zones have been observed through crossed polarizers. Such an effect has been suggested to explain part of the "overshoot" in turbidity observed in the self-assembly of sea urchin tubulin, at constant polymer mass (Hitt et al. 1988; Detrich et al. 1985). This is to be distinguished from the oscillations in microtubule mass observed more recently under different assembly conditions (Carlier et al. 1987; Pirollet

Offprint requests to: Y. Engelborghs

et al. 1987; Mandelkow et al. 1988). Spontaneous self-organization and oscillatory assembly have also been reported recently (Mandelkow et al. 1989).

The orientation of microtubules by flow has also been measured by turbidity dichroism in a Couette cell (Nordh et al. 1986). These authors found that linear dichroism develops very rapidly upon the application of the shear force. After removing the shear field, the microtubules relax back to a random orientation. The loss of dichroism is described by a sum of exponentials, with relaxation times varying between 4 and 400 s. At tubulin concentrations higher than 1 mg/ml, a stable fraction of oriented microtubules is obtained.

Flow-induced orientation of microtubules made from pure tubulin or pure tubulin plus taxol was found to be much more difficult (Wallin et al. 1986). Upon starting the flow, turbidity dichroism developed very slowly. However, rapid relaxation back to random orientation was observed within tens of seconds after ending the flow.

In this work, the kinetics of the spontaneous formation of organized regions in solution have been studied by comparing the appearance of turbidity and birefringence upon polymerization. The effect of different solvent conditions and the effect of the presence of taxol and of microtubule associated proteins (MAPs) has been studied, and the influence of different initiation conditions has been compared.

The phenomenon of spontaneous orientation appears likely to contribute to the organization of microtubules in vivo, and to have a profound influence on the kinetics of processes such as microtubule annealing, a process which occurs at high microtubule concentrations (Rothwell et al. 1986; Williams and Rone 1989).

Materials and methods

Microtubule protein (MTP) was purified from pig brains according to the method of Shelanski et al. (1973), modified as previously described (Engelborghs et al. 1977). Glycerol was used in the first cycle to increase the yield. This preparation contains mainly tubulin with about 15% of microtubule associated proteins (MAPs). Pure tubulin was obtained from microtubule protein by phosphocellulose chromatography (Weingarten et al. 1975). Its purity was checked by SDS electrophoresis. The polymerization buffer consisted of 50 m*M* MES, 70 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* NaN_3 adjusted to pH 6.4 with NaOH, the ionic strength is 0.1 M. The GTP concentration was maintained at 1 m*M* with an enzymatic regenerating system using 10 m*M* acetylphosphate and 1 unit of acetate kinase (MacNeal et al. 1977). The assembly of pure tubulin was stimulated using glycerol or dimethylsulfoxide at the concentrations indicated. Birefringence was measured at 550 nm in a thermostatted optical cuvette of 1 cm optical path and 3 mm width (Hellma, 114B-QS), placed between two crossed polarizers (polarizing sheets Ks-W 68 of E. Käsemann, Oberaudorf, Germany). The transmitted light was measured using a photomultiplier and an electronic amplifier. Unless otherwise indicated, the kinetic curves were normalized with respect to the maximum birefringence observed after a long period of equilibration.

A video system was used to study the macroscopic shape of the birefringent zones. It consisted of a CCD camera (type MO) from High Technology Holland equipped with a macro lens, an image processor (series 151) from Imaging Technology Inc. Woburn, MA, a video cassette recorder from JVC (BR-S610E) and a video timer (ForA). The CCD camera was controlled through Visilog software (Noesis, Paris).

Taxol was a gift from the Drug Synthesis and Chemistry Branch, Division of Cancer treatment, National Cancer Institute, Bethesda, MD, USA.

Results

It was expected that the formation of organized regions in solution would occur very slowly i.e. only after the tubulin had polymerized into long microtubules. In order to compare the kinetics of polymerization, as measured by turbidity, and the kinetics of the appearance of birefringence, the two measurements were made consecutively in the same cuvette, one without and one with the polarizers. The results for microtubule formation in the presence of MAPs are shown in Fig. 1. They demonstrate that the appearance of birefringence is surprisingly fast, appearing within minutes at tubulin concentrations between 1 and 3 mg/ml.

In a second series of experiments some of the different conditions commonly used for initiating microtubule assembly were compared. The most frequently used method is that of a sudden temperature rise from 4 °C to 37 °C. Upon switching between thermostat baths, using an ordinary water jacketed cuvette, a temperature gradient exists for several tens of seconds. This can be demonstrated using a temperature sensitive buffer and a dye indicator (Johnson and Borisy 1977). Such a temperature gradient might influence the direction of polymerization and promote long range organization. Therefore, a comparison was made between polymerization initiated by a temperature jump and that initiated by isothermal addition of

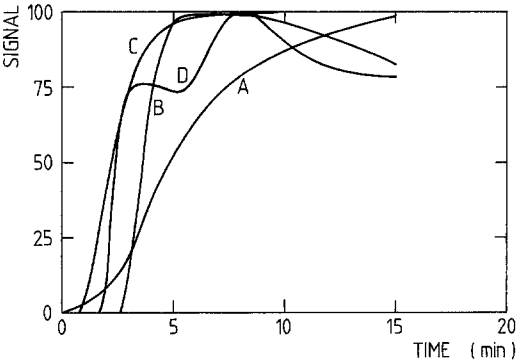

Fig. 1. Assembly of MAPs-microtubules induced by temperature jump from 4 to 37 °C at 1.0 mg/ml (A, B) and at 2.5 mg/ml (C, D) as measured by turbidity (A, C) and birefringence (B, D). All curves are plotted on a relative scale, using the maximal measured signal as reference. Limited oscillations are visible in D

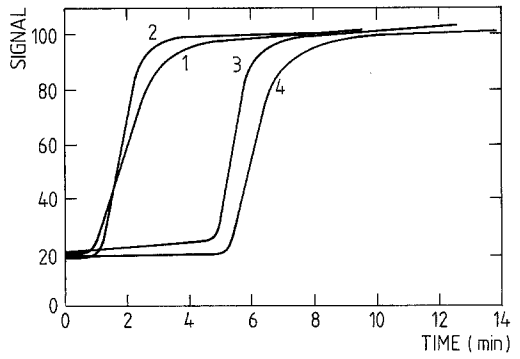

Fig. 2. Comparison of different initiation conditions. Assembly of microtubule protein at 3 mg/ml was induced by a temperature rise to 37 °C (1 = turbidity, 2 = birefringence) and by isothermal addition of GTP (3 = turbidity, 4 = birefringence) after 5 min of equilibration. All curves are plotted on a relative scale, using the maximal measured signal as reference

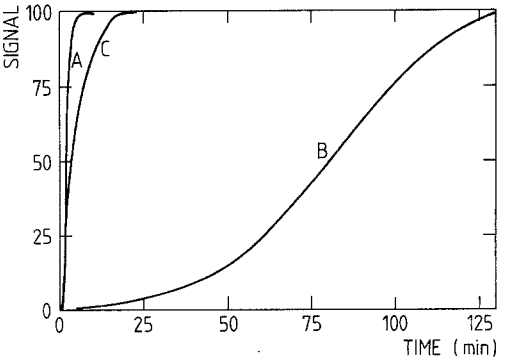

Fig. 4. Assembly of pure tubulin at 1.4 mg/ml, in polymerization buffer with 25% glycerol added. Appearance of (A) turbidity, (B) spontaneous birefringence and (C) birefringence induced by agitating the solution 2 min after nucleation. (Origin of C is immediately after agitating). All curves are plotted on a relative scale, using the maximal measured signal as reference

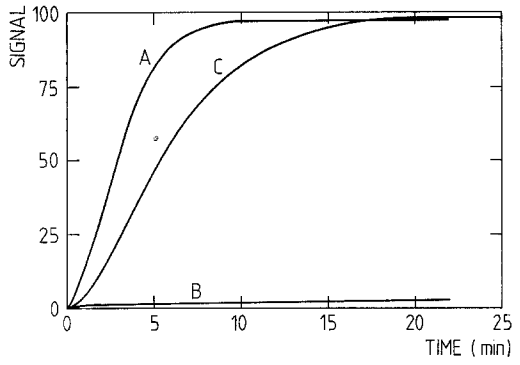

Fig. 3. Assembly of pure tubulin at 1.4 mg/ml, in polymerization buffer with 4% Me_2SO added. Appearance of (A) turbidity, (B) spontaneous birefringence and (C) birefringence induced by agitating the solution 2 min after nucleation. (Origin of C is immediately after agitating). Curves A and C are plotted on a relative scale, using the maximal measured signal as reference. Cuve B is normalized with respect to the maximum in curve C

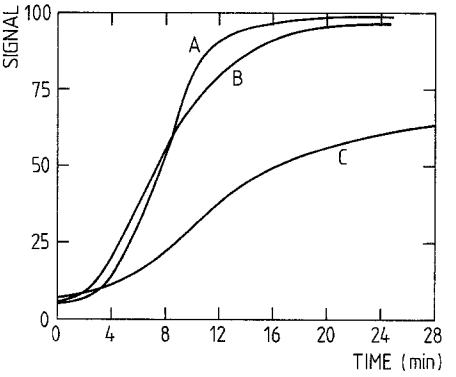

Fig. 5. Appearance of birefringence induced by agitating a solution of pure tubulin at 2.5 mg/ml in polymerization buffer, 2 min after nucleation. (A) the buffer solution contained 25% glycerol, (B) same as (A) with additional 28 μM taxol, (C) buffer with 28 μM taxol but without glycerol. Curve (A) is used as the reference. Time zero is the time of agitating

GTP at 37 °C. It was found that the appearance of birefringence was totally independent of the method used to initiate the polymerization (Fig. 2). Changing the orientation of the polarizer and the analyzer relative to the cuvette did not change the kinetics (data not shown).

In a third series of experiments, the role of MAPs was explored. Polymerization and organization of microtubules with MAPs was compared with those of pure tubulin polymerized in a buffer containing 4% dimethylsulfoxide (Fig. 3) and in a buffer containing 25% glycerol (Fig. 4).

Wallin et al. (1986) describe a very slow development of linear dichroism when microtubules are formed in the presence of taxol. We therefore repeated our experiments with pure tubulin in the presence of 28 μM taxol, with and without glycerol. As in previous cases, orientation had to be induced by agitating the solution. As Fig. 5 shows, in the presence of 25% glycerol the appearance of birefringence, after agitation, was not dependent on whether taxol was present. Even in the absence of glycerol, taxol-induced polymerization gave rise to measurable birefringence.

The appearance of birefringent zones was also followed in the same experimental system, using a video camera equipped with a macrolens. When the cuvette plus buffer solution, without protein, was subjected to a temperature jump, vertical convective flow became visible owing to refractive index changes, during the first minute. The same phenomena occurred in the presence of microtubule protein solutions, except for the rapid appearance of a few parallel spindle-shaped birefringent zones of about 0.5 cm height. Isothermal polymerizations did not show convective flow, but the shape and the appearance of the birefringent zones was very similar. During a period of about 20 min after initiation, the birefringent zones became brighter and they increased in size. Finally a single continuous transparent zone was formed. The integrity of the different zones could still be distinguished by observations at higher contrast.

Discussion

Long linear rods hinder each others motion when the covolume is larger than the free volume available in solu-

tion. For a stiff rod the covolume (V_{co}) corresponds to the disc obtained by rotating the rod through 360° about an axis perpendicular to the length of the rod and passing through its centre. The covolume is thus $\pi \cdot d\,l^2/4$, where d is the diameter and l the length (Onsager 1949). For a microtubule with a length of i μm and diameter 25 nm, the covolume can easily be calculated:

$$V_{co} = 1.963 \times 10^{-20} \times i^2 \qquad (m^3)$$

Given the number concentration (C_N) of microtubules in solution, the free volume is:

$$V_{free} = 1.661 \times 10^{-27}/C_N \qquad (m^3)$$

If the two volumes are equated, the minimal number concentration necessary for the formation of order can be estimated as

$$C_{N\,(min)} = 8.46 \times 10^{-8} \times i^{-2} \quad (M)$$

This can be converted to a minimum mass concentration (C_T) of tubulin expressed in mg/ml:

$$C_{T\,(min)} = 13.7/i$$

For the microtubules considered here, with an assumed average length between 5 and 10 μm, the critical concentration for the formation of tactoids is therefore between 1.3 and 2.6 mg/ml. This can only be considered as a rough estimate, because several other factors contribute: for example, repulsion between the rods increases the covolume. In fact it was shown theoretically that for a reversibly polymerizing system, repulsive interactions are necessary to obtain a tactoidal phase (Briehl and Herzfeld 1979). The formation of order is also facilitated in a polydisperse system, where the longer rods are preferentially arranged into parallel arrays (Flory and Frost 1978; Lekkerkerker et al. 1984). This is easily understood, since preferential packing of the longer rods in the anisotropic zones increases the entropy (freedom) of the smaller rods more efficiently.

Upon the polymerization of microtubule protein, the appearance of birefringence was observed at all concentrations tested. The normalized birefringence curve was similar to the turbidity one, except for a short delay of less than 1 min. The speed of the appearance of birefringence is unexpectedly high and suggests that the short microtubules formed in the initial stages of assembly are already orienting. The concentration dependence of the appearance of birefringence is similar to that for polymerization itself. Intuitively one would expect that fast growth of randomly nucleated microtubules might rapidly lead to entanglement and inhibit their further orientation. Therefore at high concentrations of protein, a slower development of birefringence would be expected. The experiments, however, show a relatively close correlation between polymerization and orientation at all concentrations (Fig. 1).

The observations with the video camera showed that the birefringence appears as a few spindle-like zones in solution parallel to the wall of the cuvette. With time the dark regions decrease as the zones merge. The dark regions do not move in any direction. When the organized solution is shaken, the spindle-shaped zones become very irregular and they keep their irregular shape during the 30 min of the experiment.

The two initiation methods used both showed similar correlation between polymerization and spontaneous orientation, indicating that the transient temperature gradient in the cuvette is not significantly influencing the kinetics of orientation. The orientation effect can therefore not be attributed to the fact that the walls are heated first and might preferentially induce the polymerization of microtubules parallel and close to it.

Microtubules have been shown to be easily orientable by flow (Nordh et al. 1986). With our video system, vertical convective flow was observed after a temperature jump, even in buffer solution without tubulin, owing to the temperature dependence of the refractive index. This convective flow might orient the initially formed microtubules. However, this appears unlikely, since, when assembly was initiated by the addition of GTP, no indications of flow were present, while the same spindle shaped zones were still observable in the video.

In some cases the final level of birefringence is approached in an oscillatory way (Fig. 1), although the concentrations used are far below those normally necessary to obtain oscillations in mass concentration (Carlier et al. 1987; Mandelkow et al. 1988; Pirollet et al. 1988). It is possible that these oscillations are due to the slow reorientation of birefringent zones. This might also be the reason that an apparent maximum in birefringence is sometimes observed before the maximum in turbidity.

Significantly different behaviour is found in the assembly of pure tubulin in the absence of MAPs. Orientation is observed, indicating that MAPs are not an absolute prerequisite, but the kinetics are dramatically different. The appearance of birefringence is very slow until the solution is gently agitated. The flow induced by agitation is the likely reason for the rapid development of birefringence and further polymerization probably helps to stabilize the oriented zones.

It is not clear why microtubules made in the absence of MAPs orient so much more slowly than microtubules made in their presence. The higher viscosity in 25% glycerol (=2 cp) will, of course, slow down orientation, but the slow organization was also observed in 4% dimethylsulfoxide solutions which have a viscosity similar to that of water. Differences in the kinetics of polymerization of the two systems are not very pronounced, and therefore the differences in length distributions must be rather limited. Moreover, the lengths change continuously during polymerization, so there must exist a point in time where the lengths are comparable.

A possible explanation might be the presence of more pronounced repulsive forces between the microtubules covered with MAPs. Such forces are very effective in helping the orientation of long molecules, and it was shown theoretically that they are an essential requirement for the appearance of tactoids in a polymerizing system (Briehl and Herzfeld 1979). Moreover, the absence of such repulsive forces might lead to rapid formation of an entangled network which, once formed, would orient only very slowly. Indirect evidence for the existence of strong repulsive forces in the presence of MAPs comes from the much

less dense packing of MAPs-microtubules when centrifuged (Brown and Berlin 1985, Black 1987). Microtubules made of pure tubulin are, of course, also expected to show (electrostatic) repulsive forces, since they are negatively charged at pH 6.5. Nevertheless, observations by DIC-video microscopy (unpublished observations) and in darkfield video microscopy (Bayley, personal communication) reveal that microtubules made of pure tubulin have a strong tendency to associate laterally into bundles, showing that this electrostatic repulsion is compensated by other effects. MAPs are positively charged and can overcompensate the negative charges of the tubulin monomers, resulting in a net repulsion between MAPs loaded microtubules.

Our experiments show that microtubules made from pure tubulin in the presence of taxol are also oriented by agitating the solution. Wallin et al. (1986) found that taxol-microtubules were more difficult to orient by flow in the Couette cell. This is not in conflict with our observations, as it should be realised that agitating the solution has a much more pronounced orientational effect than the very gentle forces exerted in the Couette cell.

The theory of tactoid formation predicts a slightly higher protein concentration and density in the anisotropic zones, with eventual phase separation, as observed with virus solutions after several days (Oster 1950). Owing to the inherent instability of tubulin, we have never extended our experiments over such time periods. However, the phenomenon should be speeded up at higher gravity. This was shown to be the case by Hitt et al. (1987) and by Nordén et al. (1988) using low-speed sedimentation.

The remarkably high speed of the spontaneous appearance of orientation with MAPs-microtubules will certainly influence processes which are orientation-dependent, such as annealing of microtubules. Very often the process of annealing does not show the expected dependence on the square of the number concentration of polymers (Rothwell et al. 1986). Alignment of microtubules is expected to decrease the encounter frequency of the ends. In contrast, one-dimensional diffusion of the rods along the direction of the oriented zone might be enhanced and might increase the encounter possibility, so the overall effect would be a complex function of polymer concentration.

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