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The bending of sliding microtubules imaged by confocal light microscopy and negative stain electron microscopy

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Summary

Individual microtubules can be visualised by confocal microscopy in reflection mode; when associated with a glass surface, they show up as black lines against the bright reflection from the surface. The high contrast imaging allows details of the behaviour of sliding microtubules to be studied easily.

Taxol-stabilised microtubules sliding over kinesin-coated surfaces are normally straight, but can bend into tight loops if the leading end sticks to the surface. Some remain curved after release and move in circles. In such cases, the microtubule lattice must have become stably deformed. Electron microscopy of microtubules fixed during sliding shows no gross rearrangement of the subunit lattice and indicates that microtubule bending is mainly achieved by increased twisting of the longitudinal protofilaments around the microtubule.

Keywords

tubulin; microtubule lattice; kinesin; confocal microscopy

Introduction

The sliding of microtubules over glass surfaces was discovered using video-enhanced differential interference contrast (DIC) light microscopy (Allen *et al.* 1985) and most studies of individual microtubule behaviour by light microscopy since then have employed the same technique. Dark-field microscopy, with or without video-enhancement, has also been used to obtain important new results in this field (e.g. Summers and Gibbons, 1971; Horio and Hotani, 1986). Recent developments in laser-scanning confocal microscopy (White *et al.* 1987; Amos *et al.* 1987) have improved the resolution obtainable by reflection interference microscopy to the extent that we have yet another means of observing movement of individual microtubules. Confocal reflection interference microscopy shares the advantage of DIC light microscopy that objects not close to the relevant glass surface are excluded from the image, but it produces a higher contrast than DIC; it offers the additional possibility of simultaneously imaging a fluorescence image in perfect registration with the reflection image. This report demonstrates some results obtained with just reflection imaging, followed by observation of some specimens by electron microscopy.

Materials and methods

(a) Preparation of microtubules and kinesin

Reassembled brain microtubules were stabilised with 20 μM taxol after two cycles of assembly and disassembly and MAPs were removed in medium containing high salt (Vallee, 1982). The pelleted MAP-depleted microtubules were resuspended in Pipes buffer (0.1 M Pipes-KOH, pH6.9, 1 mM EGTA) and stored in small samples in liquid nitrogen. Samples

were thawed when required and diluted in media of varying pH (0.1 M Mes, pH 6.6; 0.1 M Pipes, pH 6.9; 0.1 M Hepes, pH 7.4 or 7.8; plus 1 mM EGTA, 10 μ M taxol).

Kinesin was purified from pig brain using the protocol described by Amos (1989) and also stored in liquid nitrogen. For experiments on microtubule sliding, 5–10 μ l of kinesin, at protein concentrations of 0.1–1.0 mg ml⁻¹, were spread over the central area of a glass coverslip and incubated in a moist chamber for 1–5 min, as described by Vale *et al.* (1985). The coverslips were normally used untreated but for some experiments the surface was coated with a film of nitrocellulose, as described by Kron *et al.* (1990). The latter treatment had no obvious effect on the attachment of kinesin to the surface or to its interaction with microtubules. For observation of some samples by electron microscopy, copper grids were sandwiched between coverslip and nitrocellulose film.

5 μ l of diluted microtubule solution was added to the sample on the coverslip which was then inverted on to a slide where it was supported with strips of coverslip, also as described in detail by Kron *et al.* (1990). Additional buffer was added from either side of the coverslip, to fill the space, together with 5 mM MgATP.

(b) Confocal microscopy

A MRC500 manufactured by BioRad was used to scan the specimens through a Nikon Optiphot with a X60 Planapo objective. A 15 mW argon ion laser was used, with a narrow-band filter to isolate the 488 nm line. The standard reflection filter block was used, without polarising anti-reflection components. Laser power was varied from 1% to 100%; surprisingly, the strongest illumination used seemed to have no effect on kinesin-induced motility. However, the gain in terms of noise reduction, in increasing the power from 10% to 100%, was small. The full field that can be scanned using the $\times 60$ objective has a diameter of 200 μ m but it is possible to zoom in on any part of the field using a restricted range of scanning angles; at maximum zoom, this lens gives a field width of 25 μ m. Microtubules can be seen most clearly with a field width of 50 μ m or less. A framing rate of 1 per second was judged to be optimum both for direct viewing and for recording on video; no averaging of frames was carried out for moving specimens as this blurred the ends of microtubules.

Image contrast could be enhanced on-line at the analogue stage and after conversion to digital form. Some series of images were filed directly from the frame store on to a Winchester or optical disc. Others were fed back through the system from video-tape recordings; this gave a slight reduction in image quality but made the choice of interesting frames easier.

(c) Electron microscopy

EM grids trapped under plastic on sample-covered coverslips were lifted off, dipped briefly in wash buffer (1 mM Hepes or Pipes, 10 mM potassium acetate) and negatively stained with 1% aqueous uranyl acetate.

Results

(a) Confocal microscopy

Fig. 1 shows the appearance of microtubules in a confocal reflection interference image averaged over many frames. Microtubules just below the coverslip are visible as lines approximately 0.2 μ m thick; those very close to the glass surface appear black, others roughly half a wavelength further away appear brighter than the surface. Microtubules between these extremes would be expected to contrast less with the surface and, occasionally, grey segments can be identified.

Fig. 2 consists of a series of images extracted from single frames to illustrate the sliding of straight microtubules. Kinesin-induced movement was investigated in buffers with a range of pH values. Sliding occurred at a rate of $0.3 \mu\text{m s}^{-1}$ in 0.1 M Mes, pH6.6; at $0.4 \mu\text{m s}^{-1}$ in 0.1 M Pipes, pH6.9; at $0.6 \mu\text{m s}^{-1}$ in 0.1 M Hepes, pH7.4 or 7.8.

Microtubules were observed to bend in all these buffers; it appeared that the leading end would become stuck to the surface and pushing from the rear would produce bends near the front (e.g. Fig. 3B–D). This behaviour is similar to that described by Allen *et al.* (1985) as *fishtailing*. If the front end remained stuck, the rest of the microtubule would often rotate around it in a spiral. If the end of such a microtubule became detached again, it would sometimes re-straighten, especially in the Mes and Pipes buffers. Under conditions used in these experiments, other microtubules remained in a curved configuration and continued to move in circles (Fig. 4). The bends formed at pH values above 7.0 tended to be tighter and were more often retained after detachment. Breakage during bending (see Fig. 3) was also much commoner at higher pH. An estimate, from the confocal images, of the radii of the smallest circles seen is around $0.5 \mu\text{m}$ (i.e. a maximum curvature, before breakage, of 2 radians per micron). Figs 3 and 5 illustrate the point that initially straight microtubules can become stably bent but that stably bent microtubules can subsequently straighten again.

(b) Electron Microscopy

To discover how the microtubule lattice was accommodating to such pronounced bending, EM grids were attached under a thin film of nitrocellulose to the surface of a coverslip, on which kinesin and microtubules were combined as before. The reflection interference image was unfortunately degraded within the squares of the EM grid (presumably because the contact here between glass surface and nitrocellulose film was not close enough) but movement observed on the surface adjacent to each grid appeared to be as usual.

Normally, in the absence of kinesin, microtubule lattice angles and spacings appear to be strongly maintained. Although the longitudinal protofilaments (pfs) run straight in a 13-pf tubule, they twist slowly around the axes of the 14-pf or 15-pf tubules commonly found in reassembled microtubule samples; in each case, the twist is sufficient to preserve the lengths and angles of the standard 13-pf surface lattice (Wade *et al.* 1990). EM images such as Figs 6 and 7 show that curved microtubules remain tubular and apparently retain their subunits in basically the same helical lattice as in straight microtubules; there is no radical rearrangement of whole subunits.

Bending without damage to the lattice could be achieved by contraction of the lengths of all the subunits along the inner circumference of a curved tubule or extension of subunit spacings on the outer circumference; in other words, by contraction or extension along the protofilaments. The latter could, however, remain roughly constant in length if they were to twist around the microtubule at a suitable rate, different from that in a straight 14- or 15-pf microtubule; this would mean the lattice angles being distorted from normal, however.

The lattice parameters of curved segments of microtubule are not easily determined, since optical diffraction requires straight subjects. Analysis of such images by computer will be reported elsewhere. Meanwhile, direct inspection of the images indicates that the protofilaments do twist around curved 14-pf microtubules more rapidly than around straight 14-pf microtubules. Straight microtubules on the same grids had presumably also been sliding over the surface not long before being fixed by negative stain. Optical diffraction patterns obtained from many of these images showed complex variations in the lattice parameters along individual microtubules; these are also in the process of being analyzed. There appear to be variations in the protofilament twist angle and even small amounts of contraction (or extension) of subunit spacings along the protofilaments.

Discussion

Tubulin is an allosteric protein whose subunits appear to change conformation substantially between the disassembled and polymerized states (Ventilla *et al.* 1972). The results reported here suggest that even an intact tubular polymer is capable of holding more than one conformational state without the help of an external force; otherwise, a curved microtubule with a freed leading end would always revert to a straight form as it slid forwards. Instead, the lattice presumably drops into a local energy minimum; the number and depths of such minima may depend on conditions in the media, such as the buffer pH and the temperature. The possibility that transitions can be induced from one structural state to another in the assembled microtubule lattice has implications for the precise role of microtubules in motility and may also be relevant to their behaviour during assembly and disassembly.

The microtubules studied here were stabilized using taxol, so it is necessary to consider the question of how different they might be from 'normal' microtubules. First, there is no obvious difference in gross structure, as seen by electron microscopy, from microtubules reassembled without taxol. Second, purified kinesin appears to interact with them in the same manner as with axonemal microtubules (see Porter *et al.* 1988), which are naturally stable. The main effect of taxol seems to be enhancement of assembly at low temperature or low tubulin concentration (Schiff *et al.* 1979). Exactly how taxol exerts its stabilising effect is not known; however, it seems likely that it inhibits disassembly by binding to individual subunits and maintaining them in a conformation or state that strongly favours their polymerization. Observations such as those reported here show that it does not suppress all changes in tubulin conformation. The components that stabilise axonemal microtubules also seem to allow them to bend in any direction, so it seems reasonable to suppose that taxol-stabilized tubules are not significantly different from naturally-occurring cold-stable microtubules.

Although the two-dimensional lattice formed by tubulin during assembly is apparently quite strictly defined, it seems that the lattice may subsequently be altered in a non-elastic fashion. The lack of evidence for an 8 nm axial periodicity in the optical diffraction patterns indicates that structural changes in these sliding microtubules affect alpha- and beta-tubulin subunits equally.

Analysis of protofilament structure in three dimensions by electron microscopy and X-ray diffraction (see Beese *et al.* 1987; Amos and Baker, 1979) shows each tubulin monomer to consist of two or more structural domains. Most likely, conformational changes in tubulin, as in other proteins, depend largely on the movement of whole domains relative to one another. An important special feature is that there must be coordinated changes to whole groups of neighbouring subunits in order to distort the tubulin lattice.

It has previously been observed that flagellar microtubules can occur in stable curly and helical conformations (e.g. Costello *et al.* 1973; Amos, 1978; Miki-Noumura and Kamiya, 1976, 1979). The results reported here indicate that the tubulin lattice itself, rather than any associated protein, is primarily responsible for maintaining such configurations, though the doublet structure and presence of specialized associated proteins probably influence the precise helical conformations (Miki-Noumura and Kamiya, 1979). Bending is obviously an important part of microtubule function in flagellar motility. The ability of the tubulin lattice to assume conformations such that bent microtubules do not produce an elastic restoring force seems likely to be an important feature of flagellar beating. A cooperative change in the tubulin lattice produced by interaction with dynein might even form part of the mechanism by which the relative sliding of adjacent doublet microtubules is converted synchronously into bending.

The possible relevance of different conformational states to the function of cytoplasmic microtubules is less obvious. There is no evidence of any type of beating motion for single microtubules *in vivo*. However, preliminary observations on straight sliding microtubules suggest that variations in the twist of the lattice are not necessarily associated with obvious bending. It is not clear whether lattice distortion is a normal feature of the interaction of a microtubule with motor molecules, or simply due, in the present situation, to the attachment of kinesin to an immovable surface; but it is possible that each hydrolysis event involves structural changes in a series of neighbouring subunits. The rotation of microtubules sliding over surfaces covered with single-headed dynein (Vale and Toyoshima, 1989) may perhaps be a related phenomenon.

Finally, it may be significant that bending and breakage are more pronounced and occur more frequently at pH values above 7, a condition that is generally unfavourable for microtubule assembly *in vitro*. Possibly, in the absence of taxol or of a class of associated proteins that confer a high level of stability, some lattice changes might lead to depolymerization. Since a lattice distortion affects many individual subunits, lattice changes associated with disassembly would be highly cooperative and might possibly explain the rapid disassembly phases of dynamically unstable microtubules.

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Fig. 1. Confocal laser scanning image of microtubules, stabilized with taxol and free of associated proteins, adhering to the surface of a glass coverslip. Individual microtubules, interfering with reflection from the surface, produce black or white lines in the image. Averaged over 50 scanning frames and computer enhanced. Specimens in Pipes buffer, pH 6.9. Scale bar, 4 μm .

Fig. 2.

Series of individual scanning frames recorded on video and then played back through the confocal frame board; frames at 4 s intervals have been selected. Specimen in Hepes buffer, pH 7.4. Diffraction ripples on the right of each image come from a small central spot of light reflected from the eye-piece. White scale bar, 6 μm

Fig. 3. Confocal series, similar to Fig. 2. The long microtubule in the centre of (A) was moving in a straight line until its leading end became attached to the glass (B–C). After pronounced bending (D), the tubule broke (E). A major portion re-straightened and slid off in a straight line (F). A smaller fragment remained curved and moved in a circle (arrows in G–H). Meanwhile, a similar fragment (x) produced by an earlier event rotated until its path changed (F). Hepes buffer, pH7.4. Time intervals shown are unequal. White scale bar, 6 μ m.

Fig. 4. Confocal image series showing an arc-shaped microtubule moving around a circle. $\times$ marks the leading end. Hepes buffer, pH 7.4. 3–4 s between images. Scale bar, 3 μm .

Fig. 5. Confocal series in which an arc-shaped microtubule was seen moving initially in a circle (A–C). The original leading end stuck to the surface, producing a break (D). The rear portion became straight and slid off in a straight line (F). Hepes buffer, pH 7.4. Time intervals unequal, chosen to show particular events. Scale bar, 6 μm .

Fig. 6. Electron micrograph of a microtubule bent almost into a circle with a radius of 500 nm. Specimen in Hepes buffer, pH 7.4, before staining. Inset shows a confocal light microscope image including a similarly bent microtubule that was observed rotating. White scale bar, 100 nm for EM image, 1 μ m for confocal image.

Fig. 7. Electron micrograph of parts of a curved and a straight microtubule on a kinesin coated plastic surface. Kinesin molecules, appearing as fine rods (indicated by arrow-heads), are not easily distinguished against the irregular background of negative stain. Hepes buffer, pH 7.4. Scale bar, 100 nm.