Assembly of chick brain MAP2–tubulin microtubule protein

Analysis of tubulin subunit flux rates by immunofluorescence microscopy

Martyn F. SYMMONS* and Roy G. BURNS†

Biophysics Section, Blackett Laboratory, Imperial College of Science, Technology and Medicine, London SW7 2BZ, U.K.

A filter-based immunofluorescence-microscopy method for obtaining microtubule lengths has been developed and evaluated. Kinetic constants and mean lengths obtained show close agreement with those obtained by complementary methods applied to chick brain MAP2–tubulin microtubule protein in NaCl-supplemented buffer. The filter-based method has been used to estimate tubulin subunit flux (J_{on}) resulting from isothermal dilution of microtubule populations to various free tubulin concentrations, (c). This experimental $J_{on}(c)$ plot is significantly different from that predicted by a variety of theoretical models, but is consistent with a ‘lateral cap’ model of dynamic instability [Bayley, Schilstra & Martin (1990) *J. Cell. Sci.* **95**, 33–48] adapted to accommodate the observed vectorial GTP hydrolysis.

INTRODUCTION

Microtubules are polymers of the subunit protein tubulin with or without associated microtubule-associated proteins (MAPs), which assemble by addition of tubulin (Tu)–GTP subunits to their growing ends. The kinetic constants for these endwise interactions are affected by the presence and phosphorylation status of the MAPs, without altering the underlying kinetic mechanism (Karr *et al.*, 1980; Jameson & Caplow, 1981; Farrell & Jordan, 1982; Burns *et al.*, 1984; Farrell *et al.*, 1987).

Microtubules demonstrate two important activities: (a) spontaneous fluctuations in length due to extensive alternate periods of subunit association and dissociation (Mitchison & Kirschner, 1984; Horio & Hotani, 1986; Walker *et al.*, 1988), and (b) the assembly-dependent hydrolysis of GTP bound to the exchangeable site on the β -subunit (David-Pfeuty *et al.*, 1977).

The length fluctuation behaviour, termed ‘dynamic instability’ (Mitchison & Kirschner, 1984), occurs *in vivo* (Sammak & Borisy, 1988; Cassimeris *et al.*, 1988), and can be considered in terms of two ‘phases’ consisting of individual microtubules showing either net growth (phase 1) or rapid shortening (phase 2) (Hill, 1984), with stochastic and relatively infrequent transitions between the two. A ‘macroscopic’ steady state exists for a large population in which the phase composition does not change with time (Hill, 1986). Macroscopic modelling has been applied to the length excursions of both free-in-solution and nucleated microtubules (Hill, 1984, 1985; Bayley *et al.*, 1989) by assuming that the transition frequencies are rare compared with the subunit association and dissociation events (Hill, 1986), since ‘treadmilling’ behaviour would otherwise dominate over dynamic instability (Farrell *et al.*, 1987).

The site along the microtubule for GTP hydrolysis is controversial, having been postulated to occur immediately on assembly (coupled model; Hill & Kirschner, 1982), on being buried in the microtubule wall lattice (coupled model; Bayley

et al., 1990), at the boundary between GTP- and GDP-tubulin within the microtubule (vectorial hydrolysis; Hill & Carlier, 1983; Caplow & Reid, 1985) or at random within the microtubule (uncoupled; Carlier & Pantaloni, 1981). Numerous attempts have been made to discriminate experimentally between these competing models. Certain pre-steady-state kinetics of GTP hydrolysis have supported the vectorial rather than the random-uncoupled model (Caplow, 1986; Carlier *et al.*, 1987; Burns, 1991b), whereas others have failed to find detectable burst kinetics and have suggested that GTPase is strongly coupled to the subunit, being buried in the polymer lattice (Schilstra *et al.*, 1987; O’Brien *et al.*, 1987; Stewart *et al.*, 1990).

GTP hydrolysis is probably a necessary (but perhaps not sufficient) prerequisite for the two-phase behaviour, but an explicit connection requires a biochemical model based upon experimentally determined parameters. It has, though, generally been assumed that the growing, phase 1, microtubules terminate in Tu–GTP subunits (the GTP cap), whereas the shortening, phase 2, microtubules lack this cap, i.e. GTP hydrolysis has occurred in all the assembled subunits. A key test of the biochemical models is the change in the behaviour of assembled microtubules in response to the free [Tu–GTP], the so-called ‘ $J_{on}(c)$ plot’ (Hill & Carlier, 1983).

A complete analysis of the $J_{on}(c)$ plot requires the relevant rate constants for subunit addition and loss before and after GTP hydrolysis. Some of these have already been determined for the assembly of chick brain MAP2–tubulin in an assembly buffer containing NaCl to dissociate the MAP2–tubulin oligomers [the preceding papers (Burns, 1991a,b)]. One advantage of this experimental system is that the assembly kinetics conform to a simple pseudo-first-order reaction, and the absence of any end-to-end annealing permits the accurate determination of the association rate constant. We have therefore used this system, and the determined rate constants, to assess the key relationship between GTP hydrolysis and the length changes, using immuno-

Abbreviations used: MAP, brain microtubule-associated protein; MTP, microtubule protein (stoichiometric mix of tubulin and MAP2); GTP[S], guanosine 5’-[γ -thio]triphosphate; Tu–GTP, Tu–GDP, tubulin dimer with GTP or GDP bound to the exchangeable site; k_{+1}^{GTP} and k_{-1}^{GTP} , association and dissociation rate constants for addition and loss of Tu–GTP subunits determined during elongation phase of assembly; k_{-1}^{GDP} , the dissociation rate constant for loss of Tu–GDP subunits from uncapped microtubules; C_0^{GTP} , critical concentration of free Tu–GTP in exchange with microtubules, and equal to $k_{-1}^{GTP}/k_{+1}^{GTP}$; J_{on} , net flux of subunits on to microtubules in a population at particular [Tu–GTP], defined as positive in the assembly direction; [M], microtubule number concentration; SSpm, steady-state polymer mass; [Tu]_{total}, [Tu–GTP] given by eqn. (2) in the text; (c), free tubulin concentration in an analysis of the dependence of tubulin flux (J_{on}); d.i.c., differential interference contrast.

* Present address: Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

† To whom correspondence should be sent.

fluorescence to analyse the behaviour of microtubule populations. The direct-visualization method has distinct advantages over the turbidometric and radiometric approaches used by other workers to study $J_{on}(c)$ behaviour (Farrell *et al.*, 1983; Carrier *et al.*, 1984; Caplow *et al.*, 1985).

The observed experimental $J_{on}(c)$ behaviour for the MAP2-tubulin microtubules would be compatible with the simple vectorial hydrolysis model if the rate of re-establishing a GTP cap were comparable with the rate of subsequent subunit additions. This, however, has the effect of preventing any extensive length fluctuations. By contrast, the observed $J_{on}(c)$ plot resembles that predicted by the lateral-cap model for microtubules showing dynamic instability fluctuations (Bayley *et al.*, 1990).

MATERIALS AND METHODS

Microtubule protein and assembly

Microtubule protein was prepared as previously described (Burns & Islam, 1981) from chick brain by two cycles of assembly and disassembly, stored in liquid N_2 , and then eluted from a Sephadex G-50 column equilibrated with 100 mM-Mes/67 mM-NaCl/5 mM-EGTA/1 mM-dithiothreitol/0.5 mM-MgCl₂/0.1 mM-EDTA (adjusted to pH 6.4 with KOH; Burns, 1991a). The void-volume fraction was diluted to $\approx 20 \mu\text{M}$ -tubulin, adjusted to 100 μM -GTP/1 mM-phosphoenolpyruvate and pyruvate kinase (6 units $\cdot \text{ml}^{-1}$), and degassed. The assembly was initiated by rapid transfer to cuvettes equilibrated to 37 °C in a Beckman DU-8 recording spectrophotometer, and was monitored at A_{350} ($1 A \equiv 44 \mu\text{M}$ -tubulin).

Dilution or supplementation experiments used unassembled microtubule protein (MTP) in 100 μM -GTP and the phosphoenolpyruvate/pyruvate kinase GTP-regenerating system maintained at 4 °C. The protein was warmed to 37 °C immediately before addition of pre-assembled microtubules in order to minimize both self-nucleation of the stock and any loss in assembly-competency. The concentration of this MTP was corrected for any loss of assembly-competency of the protein.

Immunofluorescence

Aliquots (50 μl) of microtubules were added rapidly to a 100-fold excess of 37 °C-equilibrated assembly buffer containing 0.4% glutaraldehyde, and gently mixed by three inversions. The use of wide-bore (2–3 mm) tips and the gentle mixing minimized the clumping, tangling or shearing of any long microtubules. After a 10 min fixation at 37 °C, the microtubules were further diluted into assembly buffer (usually 1 in 200 for assembly or 1 in 10 for disassembly samples), and 250 μl aliquots were applied to the smooth side of track-etched polycarbonate filters (25 mm diameter; 0.4 μm pore diameter; Nucleopore Corp.; also available as Isopore from Millipore Corp.) on a microfiltration support. The filters were carefully washed with 250 μl of phosphate-buffered saline (1.5 mM-KH₂PO₄/8 mM-Na₂HPO₄/137 mM-NaCl/2.6 mM-KCl, adjusted to pH 7.3 with NaOH) containing 0.5% BSA and 0.05% (w/v) Tween-20 detergent, and air-dried. The microtubules were immunostained by sequential incubation with 1:100 of the rat anti-tubulin monoclonal YOL 34 antibody and fluorescein 5-isothiocyanate-conjugated sheep anti-rat IgG. The filters were then mounted on microscope slides in Citifluor antifade mounting medium, and examined on a Zeiss research microscope with epifluorescence condenser and appropriate filters. Fields of view were chosen on the basis of staining and appropriate concentration of microtubules for measurement, and were photographed with TMY 35 mm film (Kodak-Eastman Corp.) push-processed from 400 to 1600 ASA.

The negatives were usually printed at a final magnification of 600 $\times$. At least 200 microtubules from five fields of view were measured, the small number of microtubules that extended out of the field of view being ignored (Kristofferson *et al.*, 1982).

Other methods

The microtubule polymer number concentration ($[M]$) was calculated from the polymer protein concentration and the mean microtubule length (Farrell & Jordan, 1982), assuming a 14-protofilament lattice. Protein concentrations were determined by the method of Hartree (1972), with BSA standards, and have been expressed as mg of MTP $\cdot \text{ml}^{-1}$ or as μM -tubulin (Burns, 1991a).

Materials

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), microscopical supplies were from Agar Scientific (Stansted, Essex, U.K.), and antibodies were from Serotec (Kidderminster, Worcs., U.K.).

RESULTS

Kinetics of assembly

Assembly of the MTP commences very shortly after transfer to 37 °C, and the pseudo-first-order plot (Fig. 1a) of the instantaneous rate of assembly against the concentration of tubulin yet to assemble shows that > 85% of the assembly conforms to a single exponential phase (Burns, 1991a). The marked transition from nucleation to elongation and the linear approach towards steady-state demonstrates that there is minimal further nucleation. The slope of this linear region yields the apparent rate constant for addition of Tu-GTP ($k_{+1}^{\text{GTP}}[M]$; Burns, 1991a), whereas the end of the monotonic elongation kinetics is marked by an abrupt steepening, leading to the steady-state polymer mass (SSpm) (open symbols, Fig. 1a). This non-linearity immediately before SSpm is significantly more marked when low

Fig. 1. Pseudo-first-order plots of microtubule assembly

(a) $9.6 \pm 0.2 \mu\text{M}$ tubulin as MTP assembled ($\square$ and $\square$) with 100 μM -GTP and a GTP-regenerating system to form 5.2 μM -polymer with a pseudo-first-order rate constant ($k_{+1}^{\text{GTP}}[M]$, calculated from the points shown as $\square$) of $3.7 \times 10^{-3} \text{ s}^{-1}$. The $C_0\text{SSpm}$ was 2.0 μM -tubulin for this MTP preparation. (b) A similar concentration of tubulin as MTP assembled with ($\square$) 100 μM -GTP and a GTP-regenerating system or ($\triangle$) with 500 μM -GTP[S], seeded with sheared GTP-assembled microtubules. Regression lines, calculated from the points shown as $\square$ and $\triangle$, yield similar pseudo-first-order rate constants of 2.0 and $2.2 \times 10^{-3} \text{ s}^{-1}$ respectively. The $C_0\text{SSpm}$ with GTP[S] was decreased to 1.75 μM -tubulin.

Fig. 2. Length distributions for time course of assembly

(a) Photograph of immunostained microtubules on Nucleopore filters fixed during elongation. (b–h) Length distributions of samples removed at 2 min (b), 3 min (c), 6 min (d), 8 min (e), 8.5 min (f) after nucleation, and 5 min (g) or 35 min (h) after attaining SSpm. There was an apparent increase ($\approx 5\%$) in the mean length at steady state $[(41.4 \pm 1.2) - (43.8 \pm 1.6 \mu\text{m})]$ corresponding to a slight difference ($\approx 6\%$) between the extents of assembly of the two independent assays. Replicate samples ($9.2 \mu\text{M}$ -tubulin as MTP) were assembled to steady state. $k_{+1}^{\text{GTP}}[\text{M}] = 3.9 \pm 0.05 \times 10^{-3} \text{ s}^{-1}$, $n = 5$; SSpm = $5.75 \pm 0.03 \mu\text{M}$ -tubulin.

Fig. 3. Correlation between predicted and measured mean lengths through assembly

The mean lengths ($\pm \text{S.E.M.}$) of the distributions shown in Fig. 2 are compared with the predicted lengths based upon the measured $k_{+1}^{\text{GTP}}[\text{M}]$, the extent of assembly, and assuming that $k_{+1}^{\text{GTP}} = 40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The points on the straight line (●) are the predicted mean lengths; □, measured means ($\pm \text{S.E.M.}$, $n = 171\text{--}196$ microtubules measured/sample).

concentrations of MTP are assembled (cf. Fig. 1a with Burns, 1991a). The inflexion is consistent with an increasing fraction of the microtubules losing their terminal GTP caps and switching

from the elongating to the shortening phase, since GTPase activity also attains steady state rapidly on approach to SSpm (Burns, 1991b).

This is also supported by the kinetics of assembly with non-hydrolysable GTP analogues. In particular, the pseudo-first-order plot of microtubules assembled with $500 \mu\text{M}$ -guanosine 5'-[γ -thio]triphosphate (GTP[S]), after seeding with sheared GTP-assembled microtubules, is linear from the onset of elongation to the attainment of SSpm (Fig. 1b). Similar results have been obtained with guanosine 5'-[β - γ -imido]triphosphate and these microtubules exhibit no length redistribution under steady-state conditions.

The elongation kinetics with GTP therefore predict that the population of microtubule lengths should increase as a single Gaussian distribution until shortly before SSpm and should then broaden. Five duplicate assays (initial protein concn. $9.2 \mu\text{M}$ -tubulin) were sampled at times between nucleation and steady state. The immunofluorescence (Fig. 2a) shows clearly revealed, evenly dispersed, microtubules; electron microscopy has established that each immunofluorescence image corresponds to a single microtubule (Osborn & Weber, 1982). The series of length distributions (Figs. 2b–2f) are Gaussian-like, truncated by the origin at early time points (sample b), but becoming separated from it by subsequent elongation. Processes that could generate significant numbers of shorter microtubules, such as continued nucleation, significant dynamic instability or microtubule shearing, are not apparent. Secondly, the distribution of lengths at the approach to SSpm (sample f) is very different from the 'Poisson-like' distribution observed in some previous studies (Karr *et al.*, 1980; Farrell & Jordan, 1982; Mitchison & Kirschner, 1984; Kristofferson *et al.*, 1986), but resembles other studies (see e.g., Farrell *et al.*, 1987).

The mean microtubule length of samples taken shortly after the attainment of steady state (Fig. 2g) with those taken 20 min later (Fig. 2h) show no statistically significant difference once the slight difference in polymer mass between these cuvettes is taken into account. This may be due to the only small number of short microtubules (Martin *et al.*, 1987), since length redistribution of the chick MAP2-tubulin microtubules in the NaCl-supplemented buffer has previously been noted after shearing (Burns, 1991a). The presence of limited length excursions is, however, suggested by the increased broadening of such distributions with time (Fig. 2d versus Fig. 2e).

As the proposed modelling of the $J_{\text{on}}(c)$ data involves combining length and turbidometric measurements, it was essential to establish that the two approaches yield equivalent results. This has been tested by plotting, for the assembly of Fig. 2, the observed mean length and the predicted mean length, derived from the determined value of k_{+1}^{GTP} (Burns, 1991b) and the extent of assembly, against the concentration of assembled polymer (Fig. 3). The high correlation ($r^2 = 0.985$) confirms the validity of the immunofluorescence measurements and confirms that there is no further loss (due to dynamic instability or annealing) or gain (resulting from nucleation or spontaneous shearing) of microtubule number during the elongation phase.

The validity of the immunofluorescence measurements has also been assessed by comparing the predicted with the measured microtubule lengths for a number of assays sampled at the end of elongation. A range of values of $k_{+1}^{\text{GTP}}[\text{M}]$ was obtained by varying the initial MTP concentration and by adding seeds. The prediction of mean length was made for each assembly based on the extent of assembly and a value for k_{+1}^{GTP} of $40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Burns, 1991b) and is compared with the mean length observed by immunofluorescence (Fig. 4). The correlation shows a slight tendency for immunofluorescence to overestimate the mean

Fig. 4. Correlation between predicted and observed lengths at various initial tubulin concentrations and values of $[M]$

The predicted lengths at the end of elongation were calculated (see Fig. 3 for details) and compared with the measured values. The straight diagonal line indicates the theoretical relationship; the average of the experimental data (assuming intercepts at the origin) yields a value of k_{+1}^{GTP} of $(42 \pm 2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 17$).

Fig. 5. Microtubule length changes on dilution to below C_0^{GTP}

Microtubules were assembled with $100 \mu\text{M}$ -GTP and a GTP-regenerating system to 90% of SSpm before dilution (1:20) into the same buffer. Aliquots were removed for measurement at (a) 20, (b) 40, (c) 60 and (d) 80 s after dilution. $k_{+1}^{GTP}[M]$ was $2.45 \times 10^{-3} \text{ s}^{-1}$ and $C_0\text{SSpm} = 2.0 \pm 0.2 \mu\text{M}$ -tubulin.

lengths of populations containing numerous short microtubules. However, the derived value of k_{+1}^{GTP} was 42 ± 2 (mean $\pm$ S.E.M., $n = 17$), that is, not significantly different from that based upon bulk turbidometric measurements (Burns, 1991a).

Fig. 6. Time course of the change in mean length after dilution

The mean lengths ($\pm$ S.E.M.) of the samples shown in Fig. 5. An average of 233 microtubules were measured/sample. The mean length before dilution ($32.6 \mu\text{m}$) was estimated from the extent of assembly, $k_{+1}^{GTP}[M]$ ($2.45 \times 10^{-3} \text{ s}^{-1}$), and k_{+1}^{GTP} ($40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$).

Rate of subunit loss on dilution of microtubules assembled with GTP

The assembly of MTP ($7 \mu\text{M}$ -tubulin) was monitored spectrophotometrically until just before steady state, when it had attained 90% of SSpm ($[M] = 61 \text{ pM}$; mean length = $32.5 \mu\text{m}$), and was then diluted 1:20 into assembly buffer containing $100 \mu\text{M}$ -GTP and the GTP-regenerating system, pre-equilibrated to 37°C . Samples were then removed at 20 s intervals for determination of the length distribution (Fig. 5; samples a–d). The histograms show that each population shows a Gaussian distribution and that there is progressive shortening with time. The mean length initially decreases linearly with time (Fig. 6), consistent with end-wise depolymerization (Kristofferson *et al.*, 1980), at an initial rate of $0.23 \mu\text{m/s}$, which corresponds to a tubulin dissociation rate of $400 \text{ subunits} \cdot \text{s}^{-1}$. The rate of depolymerization, however, subsequently decreases. This departure from linearity cannot be ascribed to microtubule number loss (Johnson & Borisy, 1967), as there are few very short microtubules, even after 80 s depolymerization (Fig. 5d). The deviation could be due to an alteration in the properties of the microtubule ends by depolymerization to an intrinsically more stable core. Alternatively, it could be due to an increase in the concentration of Tu–GTP resulting from the disassembly. The magnitude of the latter effect can be calculated from the change in mean length and $[M]$ (3 pM after dilution); depolymerization of $15 \mu\text{m}$ (in $\approx 80 \text{ s}$) will increase the free tubulin concentration by $< 0.1 \mu\text{M}$, which cannot account for the observed deviation.

Microtubules which have been diluted 1:20 during elongation yield higher initial rates of disassembly (450 – 550 s^{-1}) than those diluted after steady state has been attained (350 – 450 s^{-1} ; Fig. 7). This is the first report of the rate of disassembly of microtubules diluted during the elongation phase of assembly. The similarity of curves from dilutions at later times confirms that the rate of disassembly after dilution is not markedly affected by the time at steady state (10–20 min; Carrier *et al.*, 1984) and confirms the feasibility of a $J_{on}(c)$ experiment involving multiple dilutions of microtubule populations at SSpm into various concentrations of MTP.

Two points therefore emerge from the studies of the dissociation rate on dilution. First, the observed rate is considerably greater than that observed (30 s^{-1}) during elongation (Burns, 1991b). This is consistent with vectorial or coupled models of

Fig. 7. Mean length changes after dilution at various times on the approach to and after SSpm.

Duplicate cuvettes with $10 \mu\text{M}$ -tubulin as MTP were assembled with $100 \mu\text{M}$ -GTP and a GTP-regenerating system ($k_{+1}^{\text{GTP}}[\text{M}] = 5.5 \times 10^{-3} \text{ s}^{-1}$, $C_0^{\text{GTP}} = 2.5 \mu\text{M}$). $\diamond$ and $\triangle$ are from one cuvette; $\square$ and $\circ$ from the other: $\diamond$, 7 min after nucleation, 85 % of SSpm; $\square$, 11 min after nucleation, 95 % of SSpm; $\triangle$, 10 min after time of SSpm (25 min after nucleation); and $\circ$, 20 min after time of SSpm. Error bars are S.E.M. for samples of an average of 150 measured microtubules. The free Tu-GTP concentration was estimated to be $0.12 \mu\text{M}$ after dilution at SSpm and $\approx 0.17 \mu\text{M}$ for the dilution at 85 % of SSpm.

GTP hydrolysis, but not with a slow, random, model (Carlier & Pantaloni, 1981). Secondly, there may be a previously unreported long-term change in the properties of the assembled microtubule. This time-dependency strongly suggests that the altered dissociation properties are not due to the presence of STOP proteins (Margolis *et al.*, 1986) or to the incorporation of specific tubulin isoforms (Sullivan & Wilson, 1984), but could be due to the recently observed plugs at the ends and in the lumens of reassembled microtubules (Azhar & Murphy, 1990), particularly as these plugs modulate the disassembly kinetics. Such plugs, or an alternative mechanism for the changing dissociation rate constant, may give rise to the 'pauses' observed for the disassembly of pure tubulin microtubules revealed by differential-interference-contrast (d.i.c.) microscopy (Walker *et al.*, 1988).

Assembly with GTP[S]

To demonstrate a connection between GTP hydrolysis and the increase in rate of tubulin dissociation on dilution we used the non-hydrolysable GTP analogue GTP[S]. MTP was depleted of unbound GTP by elution through a Sephadex G-50 column, and assembled with either $100 \mu\text{M}$ -GTP or $500 \mu\text{M}$ -GTP[S]. The GTP[S] assembly required to be nucleated; microtubules pre-assembled with a limiting concentration of GTP ($70 \mu\text{M}$) were sheared and added ($10 \mu\text{l}$ into $500 \mu\text{l}$ volume) to yield an apparent rate constant equivalent to that for the GTP-induced assembly (Fig. 1b). Measurement of the mean microtubule length and SSpm yielded an association rate constant ($k_{+1}^{\text{GTP(S)}}$) of $(18 \pm 2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ S.E.M., $n = 4$), i.e. $\approx 45\%$ of the GTP control. Microtubules assembled with GTP and with GTP[S] were diluted 1:20 into assembly buffer with $100 \mu\text{M}$ -GTP or $500 \mu\text{M}$ -GTP[S]. The microtubules assembled with GTP[S] were substantially more stable than those assembled

Table 1. Initial rates of subunit loss on dilution (1:20) into $100 \mu\text{M}$ -GTP or -GTP[S] of microtubules assembled to steady state with $100 \mu\text{M}$ -GTP or $500 \mu\text{M}$ -GTP[S]

Conditions		Rate (s^{-1}) (mean $\pm$ S.E.M.)
Assembly	Dilution	
$100 \mu\text{M}$ -GTP	$100 \mu\text{M}$ -GTP	340 ± 40
$500 \mu\text{M}$ -GTP[S]	$100 \mu\text{M}$ -GTP	170 ± 30
$500 \mu\text{M}$ -GTP[S]	$100 \mu\text{M}$ -GTP[S]	175 ± 20

Fig. 8. Mean length changes with time as a function of the free tubulin concentration

Examples of the change in microtubule length ($\pm$ S.E.M.) on dilution (1:50) of microtubules assembled to steady state into $0.15 \mu\text{M}$ ($\circ$), $1.82 \mu\text{M}$ ($\square$) and $4.12 \mu\text{M}$ ($\triangle$)-Tu-GTP, $100 \mu\text{M}$ -GTP and a GTP-regenerating system. The microtubules ($5.2 \mu\text{M}$ -tubulin as MTP) were initially assembled to steady state ($k_{+1}^{\text{GTP}}[\text{M}] = 3.7 \times 10^{-3} \text{ s}^{-1}$; SSpm = $2.3 \pm 0.2 \mu\text{M}$ -Tu-GTP) with $100 \mu\text{M}$ -GTP and the GTP-regenerating system.

with GTP (Table 1), irrespective of whether the dilution buffer contained GTP or GTP[S]. This enhanced stability is also indicated by a small decrease in estimated $C_0\text{SSpm}$ (from 2.0 to $1.75 \mu\text{M}$ -tubulin) and by the absence of any inflexion in the assembly kinetics on the approach to SSpm (Fig. 1b).

Subunit addition and loss as a function of the free Tu-GTP concentration

The rate of subunit addition and loss is expected to be dependent on the free [Tu-GTP] in a non-linear fashion (Hill & Carlier, 1983; Hill, 1986). This was investigated by measuring the change in the mean microtubule length as a function of time after dilution of pre-assembled microtubules into fixed concentrations of Tu-GTP. The microtubules were initially assembled to steady state at 37°C , and then diluted (1:50) into the appropriate concentration of Tu-GTP. A number of precautions were taken to obtain valid kinetics.

(a) The microtubule population was diluted only after 10 min at SSpm (see Fig. 7).

(b) The stock Tu-GTP was stored at 4°C in $100 \mu\text{M}$ -GTP and the GTP-generating system until use. At the end of the experiment, the stock Tu-GTP was assembled to steady state and compared with the initial assembly in order to calculate the decrease in the assembly-competency of the protein. The loss of

Fig. 9. Experimental $J_{on}(c)$ plot

Initial rate of change of the mean length ($\pm$ S.E.M.) as a function of the concentration of free assembly-competent Tu-GTP. This involves slight corrections to the concentrations given initially present in dilution assay (e.g. Fig. 8 values). The point at SSpm ($J_{on} = 0$) was the experimentally determined critical concentration. The data point at $5.3 \mu\text{M}$ -tubulin is likely to be underestimated, owing to some shearing of the particularly long microtubules formed in this assay. The closed symbols and fitted line are calculated values (see the text), whereas the straight line intercepting at 20 s^{-1} is the predicted elongation rate for the subpopulation of microtubules with a terminal GTP cap, based upon the known value of k_{+1}^{GTP} ($40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$).

this competency is slow at 4°C , and is linear with time. This permitted the concentration of assembly-competent Tu-GTP to be accurately calculated for each assay.

(c) The free Tu-GTP concentration was also corrected for the increase in concentration resulting from the dilution-induced disassembly of the microtubules, using the value of $[M]$, estimated from the assembly kinetics, and the observed extent of disassembly.

(d) The sampling times of the parallel dilutions were chosen to give approximately the same change in mean length ($\approx 15 \mu\text{m}$), and consequential increase in free Tu-GTP. Calculation of the dissociation rates was restricted to the initial linear portion of the disassembly kinetics (see Fig. 6).

(e) The free Tu-GTP concentration was corrected for the amount of residual protein resulting from the dilution of the $C_0\text{SSpm}$. This value ($2.3 \pm 0.2 \mu\text{M}$ -tubulin) was determined by plotting the extent of assembly at steady state of increasing concentrations of MTP and extrapolating to the ordinate axis (Zackroff *et al.*, 1982), and was consistent with the predicted value determined by measuring the mean lengths of the assembled microtubules.

Immunofluorescence measurements of steady-state microtubules diluted into different concentrations of Tu-GTP, corrected for the assembly-competency of the protein, either elongate or shorten with time (Fig. 8). Plotting the initial rates as a function of the assembly-competent Tu-GTP concentration yields the $J_{on}(c)$ plot. This plot is non-linear (Fig. 9), intercepting the ordinate at a value approaching k_{-1}^{GDP} , whereas the curve is convex between $C_0\text{SSpm}$ and this intercept, i.e. intermediate rates are greater than predicted by a straight line. This contrasts with earlier predictions (Carlier *et al.*, 1984), which concluded that this portion of the steady-state rate of GTP hydrolysis (SS-GTPase) $J_{on}(c)$ plot would be concave. In addition, the slope of

the $J_{on}(c)$ plot around and below $C_0\text{SSpm}$ is higher than the extrapolated value of k_{+1}^{GTP} measured for the elongating microtubules. This confirms that there is a discontinuity in the rate of assembly on the approach to SSpm, consistent with the observed inflexion (Fig. 1a). Finally, the observed $J_{on}(c)$ plot approaches k_{-1}^{GDP} asymptotically, with no evidence for an inflexion at low Tu-GTP concentrations between a rate of subunit loss equal to k_{-1}^{GDP} and the progressively decreasing rate. This indicates that microtubules terminating in Tu-GDP subunits must be readily recapped, even at very low free Tu-GTP concentrations.

DISCUSSION

The observed k_{+1}^{GTP} during elongation from immunofluorescence estimates of $[M]$ (Figs. 3 and 4) is larger than any value previously determined for pure tubulin microtubules, but is comparable with previously reported values for MAP-tubulin microtubules (Burns, 1991a). The observed value is, however, twice as high as that reported for the subpopulation of pure tubulin microtubules with a terminal GTP cap measured by d.i.c. microscopy (Walker *et al.*, 1988). The inhibition of dynamic instability by MAPs would be expected to yield a microtubule population which, as observed (Fig. 2), elongates monotonically at a high rate (Bré & Karsenti, 1990).

The value for k_{-1}^{GTP} (30 s^{-1} ; Burns, 1991a) depends crucially on an estimate of C_0^{GTP} , but is comparable with the value reported for pure tubulin microtubules (Walker *et al.*, 1988). Previous measurements of dissociation rates after GTP hydrolysis (see, e.g., Karr *et al.*, 1980; Jameson & Caplow, 1981; Farrell & Jordan, 1982; Schilstra *et al.*, 1987) have examined the dissociation of Poisson-like distributions of short microtubules, often produced by shearing. Such determinations are highly dependent upon the estimation of initial microtubule number and on the exact length distribution. Furthermore, the tips of sheared microtubules may not be in the steady-state average configuration (Chen & Hill, 1983). The immunofluorescence approach has sought to determine the initial rate of subunit loss under non-perturbing conditions, working at values of $[M]$ which result in minimal re-addition of the dissociated subunits. The observed value ($350 \pm 50 \text{ s}^{-1}$) is significantly lower than that reported for pure tubulin microtubules (1650 s^{-1} ; Walker *et al.*, 1988), even accounting for the difference in the Mg^{2+} concentration (O'Brien *et al.*, 1987; Gal *et al.*, 1988), and this is attributed to the presence of MAP2.

The non-hydrolysable analogue GTP[S] was reported as not to be able to support microtubule assembly (Hamel & Lin, 1984). This effect is due to the suppression of nucleation, as microtubules do elongate after seeding (Fig. 1b) with incorporation of $[^3\text{H}]$ - or $[^{35}\text{S}]$ -GTP[S] (Roychowdhury & Gaskin, 1986; Stewart *et al.*, 1990). The properties of the microtubules assembled with $500 \mu\text{M}$ -GTP[S] (Fig. 1b and Table 1) provide strong evidence that the increase in the dissociation rate constant after assembly of microtubules with GTP is a direct consequence of GTP hydrolysis. This is also indicated by the failure of microtubules assembled with guanosine 5'-[$\beta\gamma$ -imido]triphosphate to exhibit length redistribution (Burns, 1990). This therefore raises the question about the exact relationship between the assembly-dependent GTP hydrolysis and the conformational change within the tubulin lattice indicated by the enhancement of the dissociation rate constant.

This relationship is restricted by the observation that GTP hydrolysis occurs as a vectorial process (Carlier *et al.*, 1987; Burns, 1991b). A kinetic scheme (Scheme 1) of the vectorial-hydrolysis model treats the microtubule as a single-stranded

Scheme 1. Vectorial-hydrolysis model showing parameters controlling rates

The numerical values used in this modelling are:

Parameter	Value	Reference
k_{+1}^{GTP}	$40 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	Burns, 1990a; the present paper
k_{-1}^{GTP}	20 s^{-1}	
k_{-1}^{GDP}	300 s^{-1}	
k_{cat}	175 s^{-1}	The present paper
k_{+1}^{cap}	$(0.4-10) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	
k_{-1}^{cap}	20 s^{-1}	

Values of k_{-1}^{GTP} and k_{cat} slightly different from the experimentally determined values [80 s^{-1} (Burns, 1991a); 200 s^{-1} (Burns, 1991b)] have been used in order to yield the best fit to the experimental $J_{on}(c)$ plot (Fig. 9).

single-ended polymer (Hill, 1986), with GTP hydrolysis only occurring in subterminal subunits. Scheme 1 includes a further simplification in that the free Tu is assumed to be all Tu-GTP, consistent with the inclusion of the GTP-regenerating system, and does not include the proposal (Caplow & Reid, 1985) that there is both productive and non-productive subunit addition. The experimentally determined rate constants are used in conjunction with the kinetic equations of Hill (1986) to model the $J_{on}(c)$ plot.

The model predicts that the addition of Tu-GTP to a microtubule end terminating in a Tu-GTP subunit is governed by:

$$J_{on} = k_{+1}^{GTP} [\text{Tu-GTP}] - k_{-1}^{GTP} \quad (1)$$

The size of the terminal GTP cap is governed by this addition/loss and by the rate constant for the GTP hydrolysis, k_{cat} . Analysis of pre-steady-state kinetics shows, on the basis of vectorial GTP hydrolysis, that k_{cat} equals 200 s^{-1} (Burns, 1991b). There will be a minimum Tu-GTP concentration for which this cap is of finite size ($[\text{Tu}]_{bal.}$), defined as:

$$[\text{Tu}]_{bal.} = (k_{-1}^{GTP} + k_{cat}) / k_{+1}^{GTP} \quad (2)$$

All the microtubules will terminate in Tu-GTP subunits at Tu-GTP concentrations above $[\text{Tu}]_{bal.}$, and will have growth properties determined by eqn. (1), whereas below $[\text{Tu}]_{bal.}$ there will be a stochastic probability that some of the microtubules may lack a terminal GTP cap. Such microtubules will dissociate at a rate governed by k_{-1}^{GDP} unless they are recapped. This recapping is demonstrated by the constant mean microtubule length of unsheared microtubules maintained at SSpm (Figs. 2d and 2e).

The rate of subunit addition during elongation should therefore deviate from a simple pseudo-first-order reaction when the $[\text{Tu-GTP}]$ declines below $[\text{Tu}]_{bal.}$. Such an inflexion is observed (Figs. 1a and 1b), although at a slightly lower free $[\text{Tu-GTP}]$ than that predicted by eqn. (2) ($4.75 \mu\text{M}$). This difference is partly due to the difficulty in precisely defining the inflexion point and also to the small, and constant, rate of turbidity increase which is not associated with microtubule assembly.

At SSpm, the rate of recapping and subsequent subunit addition exactly equals the net disassembly rate, without (see Figs. 2d and 2e) any change in microtubule number, and the free tubulin concentration equals $C_0\text{SSpm}$. It is important to note that this does not correspond to $[\text{Tu}]_{bal.}$, as has sometimes been assumed (Carrier *et al.*, 1984), and that the slope of the line in the range $C_0\text{SSpm} < [\text{Tu-GTP}] < [\text{Tu}]_{bal.}$ is always steeper than predicted by k_{+1}^{GTP} (Bayley *et al.*, 1990). This is due to the increasing occurrence of the unstable Tu-GDP lattice at microtubule tips as $[\text{Tu-GTP}]$ falls (Burns, 1991b). By contrast, a similar effect was interpreted in terms of a lower k_{+1} (Carrier *et al.*, 1987). This error led them to suggest a C_0^{GTP} which is higher than that at steady state, which is both thermodynamically unlikely (Hill & Kirschner, 1982) and which conflicts with the extrapolated behaviour of capped microtubules observed by d.i.c. microscopy (Walker *et al.*, 1988).

The shape of the $J_{on}(c)$ plot (Fig. 9) is convex, in contrast with the predicted concave form (Hill & Carrier, 1983). A similar convex form has been reported (Carrier *et al.*, 1984), derived from the turbidometric kinetics of disassembly on dilution. This study argued that the apparent discrepancy between the predicted and observed curves was due to the population not having attained a true SS-GTPase during the measurement of the rate of disassembly. A Monte Carlo simulation was used to argue that, had the population actually attained SS-GTPase, the graph would have been concave. They further argued that the observed shape was consistent with pre-steady state transients obtained about 5 s after dilution, i.e. before the population rate of disassembly had accelerated to that characteristic of SS-GTPase (Chen & Hill, 1983).

This explanation can be discounted in the present study, since the rates of disassembly were obtained over a substantial time period (60 s), without any loss in microtubule number and with no apparent acceleration, which would indicate the SS-GTPase had not yet been attained (Chen & Hill, 1983). Indeed, the initial rate of disassembly was higher when pre-steady-state microtubules were diluted (Fig. 7).

We have therefore modelled the $J_{on}(c)$ plot (Scheme 1 and Fig. 10) by assuming that SS-GTPase has been attained. In contrast with transients which require Monte Carlo simulation (Chen & Hill, 1983), there are analytical solutions to the formation and loss of GTP-Tu subunits depending on the free $[\text{GTP-Tu}]$. The assumption of SS-GTPase at any fixed $[\text{Tu-GTP}]$ allows application of the principle of microscopic reversibility to be applied to the kinetic diagram of Scheme 1 (Hill, 1989). This allows the equations describing the steady-state concentrations of the various capped forms at chosen $[\text{Tu-GTP}]$ to be obtained. The equations for the vectorial-hydrolysis model were presented by Hill (1986), with GTP hydrolysis only occurring in subterminal subunits. We obtain the calculated $J_{on}(c)$ behaviour by using a microcomputer program to first calculate the concentrations of the various capped and uncapped forms over the range of $[\text{GTP-Tu}]$ of interest (specifically $0 < [\text{GTP-Tu}] < [\text{GTP-Tu}]_{bal.}$) and then to sum the subunit flux of these various contributing forms to give the average for the whole population at SS-GTPase. This average flux is the J_{on} for the population, and calculating J_{on} for selected Tu-GTP concentrations yields the $J_{on}(c)$ plot (Fig. 10).

The overall shape of the plot below $[\text{Tu}]_{bal.}$, and especially below $C_0\text{SSpm}$, is highly dependent upon the kinetics of recapping, that is, the formation of the critical nucleus at the end of a Tu-GDP microtubule such that further subunit addition is governed by k_{+1}^{GTP} . We define the rate constants of subunit addition and loss involved in recapping as k_{+1}^{cap} and k_{-1}^{cap} . Neither of these constants can be experimentally determined by the current procedures, although an estimate of their ratio

Fig. 10. Theoretical $J_{on}(c)$ plots

Predicted curves of the relationship between the rate of subunit addition or loss as a function of the concentration of free, assembly-competent Tu-GTP, assuming values of k_{-1}^{cap} of (a) 0.01, (b) 0.1, and (c) 0.25 that of k_{+1}^{GTP} and that k_{-1}^{cap} equals k_{-1}^{GTP} , and the kinetic mechanism shown in Scheme 1.

($k_{-1}^{cap}/k_{+1}^{cap}$) has been obtained by monitoring the behaviour of individual microtubules by d.i.c. (Walker *et al.*, 1988). We have consequently followed the approach of Hill (1986) and have set k_{-1}^{cap} to equal k_{-1}^{GTP} and have then altered the kinetics of cap nucleation by varying k_{+1}^{cap} . We have calculated the $J_{on}(c)$ plot setting k_{+1}^{cap} to be (a) $\frac{1}{100}$, (b) $\frac{1}{10}$ and (c) $\frac{1}{4}$ the value of k_{+1}^{GTP} . The shape of the $J_{on}(c)$ plot gradually alters from being strongly concave (see, e.g., Fig. 10, curve a) to being convex (curve c). This suggests, subject to the other limitations of the modelling, that the recapping of the chick brain MAP2-tubulin microtubules is highly efficient, consistent with the asymptotic approach of the $J_{on}(c)$ plot towards k_{-1}^{GDP} (see the Results section) and with earlier results based upon the disassembly of pure tubulin microtubules on dilution into GTP, excess GDP and into a GTP-hydrolytic system (Caplow *et al.*, 1985).

This modelling is based on the assumption that each of the 13 protofilaments can be considered independently and one consequence of k_{+1}^{cap} approaching k_{+1}^{GTP} is that only minimal length excursions would be permitted. Although this may be appropriate for the MAP2-tubulin microtubules, with their minimal length excursions (Horio & Hotani, 1986; Burns, 1990), it would be advantageous to seek an explanation for the observed $J_{on}(c)$ plot which has equal validity for pure tubulin and MAP2-tubulin microtubules.

A variety of Monte Carlo simulations (see, e.g., Chen & Hill, 1985; Bayley *et al.*, 1990) have considered the consequences of multiple sites of subunit addition and loss, corresponding to the 13 protofilaments or to the 5-start or 8-start structural lattices. Such simulations have led to the formulation of a 'lateral-cap model' of dynamic instability (Bayley, 1990; Bayley *et al.*, 1990). One particular feature of the lateral-cap model, required to accommodate the evidence that pure tubulin microtubules lack a detectable GTP cap, is that GTP hydrolysis is postulated to be induced in the terminal Tu-GTP subunit by the addition of an incoming subunit. This restriction is not required for the rapidly elongating MAP2-tubulin microtubules, since there is a detectable GTP cap (Burns, 1991b), but may apply under steady-state conditions. A second feature is that the protofilaments are not considered independently, that is, GTP

hydrolysis depends upon both longitudinal and lateral interactions between the incoming Tu-GTP and subunits at the microtubule end. Significantly, the $J_{on}(c)$ plots for such lateral-cap models are convex about SSpm (Bayley *et al.*, 1990), and closely resemble the experimental data (Fig. 9). Whereas the lateral-cap model predicts a rate of subunit loss equal to k_{-1}^{GDP} at low Tu-GTP concentrations, such that the $J_{on}(c)$ plot is sigmoidal, this is not experimentally observed (Fig. 9), owing to the efficient recapping at low Tu-GTP concentrations (see the Results section). The lateral-cap model consequently offers the best description of the observed kinetics for the chick brain MAP2-tubulin microtubules, subject to the limitation that GTP hydrolysis can be uncoupled from subunit addition during the exceptionally fast elongation phase.

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