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Microtubule assembly kinetics

Changes with solution conditions

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The assembly kinetics of microtubule protein are altered by ionic strength, temperature and Mg^{2+}, but not by pH. High ionic strength (I0.2), low temperature ($T < 30$ °C) and elevated Mg^{2+} ($\geqslant 1.2$ mM) induce a transition from biphasic to monophasic kinetics. Comparison of the activation energy obtained for the fast biphasic step at low ionic strength (I0.069) shows excellent agreement with the values obtained at high ionic strength, low temperature and elevated Mg^{2+}. From this observation it can be implied that the tubulin-containing reactant of the fast biphasic event is also the species that elongates microtubules during monophasic assembly. Second-order rate constants for biphasic assembly are $3.82(\pm 0.72) \times 10^7$ $M^{-1} \cdot s^{-1}$ and $5.19(\pm 1.25) \times 10^6$ $M^{-1} \cdot s^{-1}$, and for monophasic assembly the rate constant is $2.12(\pm 0.56) \times 10^7$ $M^{-1} \cdot s^{-1}$. The microtubule number concentration is constant during elongation of microtubules for biphasic and monophasic assembly.

INTRODUCTION

Assembly *in vitro* of microtubule protein, composed of tubulin and microtubule-associated proteins (MAPS), is kinetically characterized by three general features: lag, elongation and equilibrium processes. Early kinetic analysis of elongation or growth revealed that process to be a single pseudo-first-order event (Bryan, 1976; Johnson & Borisy, 1977). Such monophasic kinetics were explained by addition of tubulin dimer to microtubule ends and a rejection of the involvement of tubulin rings in the elongation process (Johnson & Borisy, 1977).

In subsequent work, contrary results were reported with the observation of two pseudo-first-order kinetic events (Barton & Riazi, 1980; Kumar, 1981; Bayley *et al.*, 1983). The biphasic result was accounted for by invoking the participation of oligomeric forms of tubulin in the fast growth step (Barton & Riazi, 1980; Bayley *et al.*, 1983). During the first few minutes of assembly, the major source of tubulin incorporated into microtubules comes from rings (Pantaloni *et al.*, 1981). From X-ray kinetic studies of assembly it was learned that rings dissociate into protofilament-like fragments (Mandelkow *et al.*, 1980). These fragments are incorporated into microtubules and do not dissociate further into dimers (Mandelkow *et al.*, 1980). Rings of tubulin have been extensively characterized (Kirschner *et al.*, 1974; Vallee & Borisy, 1978; Scheele & Borisy, 1978; Voter & Erickson, 1979; Mandelkow *et al.*, 1983); they are composed of tubulin and MAPs (Kirschner *et al.*, 1974; Vallee & Borisy, 1978). Tubulin devoid of MAPs exhibits monophasic assembly kinetics, but addition of MAPs to tubulin resulted in biphasic kinetics (Kumar, 1981).

The observation of monophasic kinetics by some workers and biphasic kinetics by others when microtubule protein assembles into microtubules has not been satisfactorily explained heretofore. In the present work we demonstrate that ionic strength (I), Mg^{2+} concentration and temperature strongly influence the type of assembly kinetics displayed by microtubule protein.

EXPERIMENTAL

Assembly buffers contained 1 mM-GTP and were either 50 mM-PEMg, pH 6.9, 100 mM-PEMg, pH 6.9, or 100 mM-MEMg, pH 6.5. PEMg refers to 0.05 M or 0.1 M-Pipes with 1 mM-EGTA and 0.3 mM-Mg^{2+}. MEMg is 0.1 M-Mes with 1 mM-EGTA and 0.3 mM-Mg^{2+}. Bovine brain microtubule protein was prepared by two cycles of assembly and disassembly in 0.1 M-PEMg, pH 6.9, with 1 mM-GTP and 0.1 mM-phenylmethylsulphonyl fluoride. DMSO (10%) was included during the assembly steps. Protein concentrations were measured by the method of Bradford (1976), with bovine albumin as the standard. The Philips 300 instrument was used for electron microscopy of microtubules negatively stained with 1% uranyl acetate. The magnification was 3300× for length measurements of microtubules on carbon-coated Formvar grids of 150 mesh. For length measurements, samples (50 and 100 μl) were taken at various times throughout assembly, which was monitored turbidimetrically. The samples were diluted 1:10 with 4 M-glycerol in the appropriate buffer at 37 °C to give a final protein concentration of 0.1 mg/ml.

All assemblies were monitored turbidimetrically at 350 nm in the presence of 1 mM-GTP at the appropriate temperature by using a Perkin–Elmer 552 recording spectrophotometer with thermostatically controlled cell holders. Cold assembly mixtures were placed into prewarmed cuvettes to initiate assembly.

RESULTS

Kinetic analysis

The data for the growth phase of assembly was fitted to one of two equations where A_t is absorbance at time t, A_∞ is the final absorbance value, and A_1 and A_2 are amplitudes representing $(A_\infty - A_0)$ for the fast and slow biphasic reactions respectively. Eqn. (1) describes pseudo-first-order reactions or monophasic kinetics and

Abbreviations used: PEMg, Pipes/EGTA/$MgSO_4$; MEMg, Mes/EGTA/$MgSO_4$; MAPs, microtubule-associated proteins; DMSO, dimethyl sulphoxide; MTN, microtubule number concentration; C_c, critical concentration.

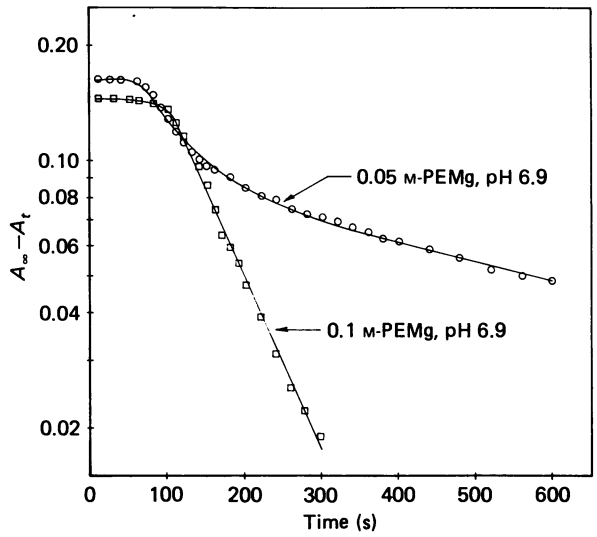

Fig. 1. Effect of ionic strength (*I*) on the kinetics of assembly

Assembly conditions were 1 mM-GTP, 1 mM-dithiothreitol, 37 °C and 0.83 mg of microtubule protein/ml. Lines were plotted by using $A = 0.169 \pm 0.003$, $k = 0.0103 \pm 0.0004$, $A_\infty = 0.177 \pm 0.002$ for 0.1 M-PEMg, pH 6.9 (*I*0.212) and by using $A_1 = 0.0763 \pm 0.0060$, $k_1 = 0.0158 \pm 0.0016$, $A_2 = 0.0897 \pm 0.0252$, $k_2 = 0.0012 \pm 0.00067$, $A_\infty = 0.211 \pm 0.031$ for 0.05 M-PEMg, pH 6.9 (*I*0.106).

eqn. (2) describes two parallel pseudo-first-order reactions or biphasic results. Goodness-of-fit was based on residual mean square, the serial coefficient and visual observation.

$$A_t = A_\infty - (A_\infty - A_0)e^{-kt} \quad (1)$$

$$A_t = A_\infty - A_1 e^{-k_1 t} - A_2 e^{-k_2 t} \quad (2)$$

Zero time for growth was taken at the end of nucleation and was evaluated as described by Bayley *et al.* (1985); in consequence, the amplitudes for biphasic kinetic growth were the same order of magnitude. If zero time was taken at the beginning of nucleation, the rate constant values were little changed, but amplitude values were affected. The non-linear regression program of the BMDP Statistical Software (University of California, 1985) was used to calculate A_∞, the amplitudes and the rate constants. Calculated A_∞ values agreed with observed values within experimental error. Use of calculated A_∞ should remove any bias toward kinetic type that might arise from estimated values.

Effect of ionic strength and pH

Three sulphonate buffers, varying widely in ionic strength [100 mM-MEMg, pH 6.5 (*I*0.069), 50 mM-PEMg, pH 6.9 (*I*0.106) and 100 mM-PEMg, pH 6.9 (*I*0.212)] were used in the present investigation. Ionic-strength calculations were based on the sulphonate salts, and where appropriate, on phosphate salts and KCl; contributions by $MgSO_4$ and EGTA were negligible and constant for all solutions, except where specified.

The type of kinetics (monophasic or biphasic) observed in Pipes buffer at pH 6.9 depended on the ionic strength. Monophasic kinetics were obtained at *I*0.212 and biphasic kinetics between *I*0.05 and 0.189 (Fig. 1). The results of typical experiments are summarized in Table 1. The observed rate constant for the fast biphasic step exhibits a maximum of 0.02 s^{-1} from *I*0.1 to *I*0.16; above *I*0.16 it decreases toward the observed rate constant for monophasic kinetics at 0.01 s^{-1}. The slow biphasic rate constant had a small positive dependence on *I*. The ratio of k_1/k_2 decreased from 12 at *I*0.106 to 1 at *I*0.212. The ratio of amplitudes for the two biphasic steps was close to 1 from *I*0.08 up to *I*0.212, where monophasic kinetics are observed. Below *I*0.08, the amplitude for the fast step decreased in value precipitously. For any given solution condition, some experimental variation in the value of rate constants was observed. At pH 6.9, average values of observed rate constants and their standard errors were computed to be as follows: k_1, $= 2.46(\pm 0.22) \times 10^{-2}$ s^{-1}; k_2, $2.39(\pm 0.19) \times 10^{-3}$ s^{-1} ($n = 5$ at *I*0.106); k, 9.52 $(\pm 1.10) \times 10^{-3}$ s^{-1} ($n = 9$ at *I*0.212).

To evaluate the generality of the effect of Pipes, the ionic strength was varied with KCl. The basic buffer was 50 mM-PEMg, pH 6.9 (*I*0.106), and KCl was added to this buffer to raise the ionic strength. Biphasic assembly kinetics were found with the observed fast rate constant decreasing almost linearly from *I*0.106 to *I*0.166, at which point the kinetics became monophasic.

The maximum extent of reaction ($A_\infty - A_0$) exhibited a maximum plateau between *I*0.1 and 0.2 for Pipes buffer, pH 6.9. At *I*0.3 and 0.4, no assembly was observed with a protein concentration of 0.83 mg/ml. Similarly, Himes *et al.* (1979) reported the inhibitory nature of intermediate concentrations of Pipes and the dependence of inhibition on protein concentration. When ionic strength was

Table 1. Effect of ionic strength on the kinetics of assembly

I values were achieved by varying the Pipes concentration from 25 mM to 100 mM at a constant pH 6.9. Assembly conditions were 1 mM-GTP, 1 mM-dithiothreitol, 0.3 mM-$MgSO_4$, 37 °C and microtubule protein (0.83 mg/ml). The observed rate constants, k_1 and k_2, are reported with the S.E.M. for the fit to the experimental data.

$10^2 \times k_1$ (s^{-1})	$10^3 \times k_2$ (s^{-1})	$10^2 \times A_1$	$10^2 \times A_2$	*I*	k_1/k_2
1.32±0.30	1.18±1.05	2.37±0.56	5.08±2.00	0.053	11.2
1.88±0.11	1.45±0.24	9.80±0.26	7.66±0.33	0.079	13.0
2.04±0.18	1.66±0.54	11.8±0.4	7.86±0.73	0.106	12.0
2.09±0.18	3.90±0.44	11.0±0.6	8.28±0.62	0.159	5.4
1.44±0.13	3.31±0.86	11.3±10.0	5.16±0.98	0.189	4.4
1.03±0.30	*	17.5±0.3	*	0.212	–

* Monophasic kinetics.

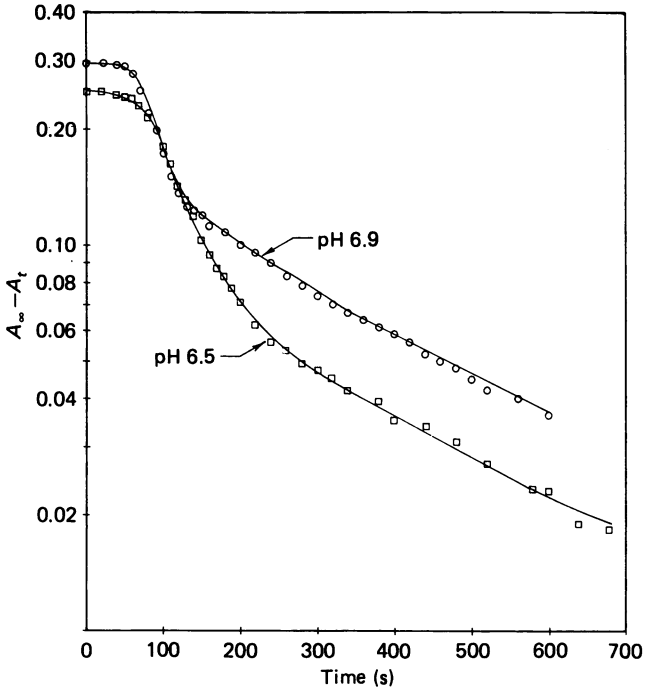

Fig. 2. Effect of pH on the kinetics of assembly

An I value of 0.106 was obtained at pH 6.9 with 0.05 M-PEMg and at pH 6.5 with 0.05 M-PEMg plus 0.024 M-KCl. The microtubule-protein concentration was 1.06 mg/ml; see Fig. 1 for other assembly conditions. Lines were plotted by using $A_1 = 0.412 \pm 0.018$, $A_2 = 0.149 \pm 0.002$, $k_1 = 0.0308 \pm 0.0012$, $k_2 = 0.00245 \pm 0.00013$ and $A_\infty = 0.352 \pm 0.002$ for pH 6.9, and $A_1 = 0.446 \pm 0.016$, $A_2 = 0.0825 \pm 0.0026$, $k_1 = 0.0198 \pm 0.0006$, $k_2 = 0.00228 \pm 0.00007$ and $A_\infty = 0.300$ for pH 6.5.

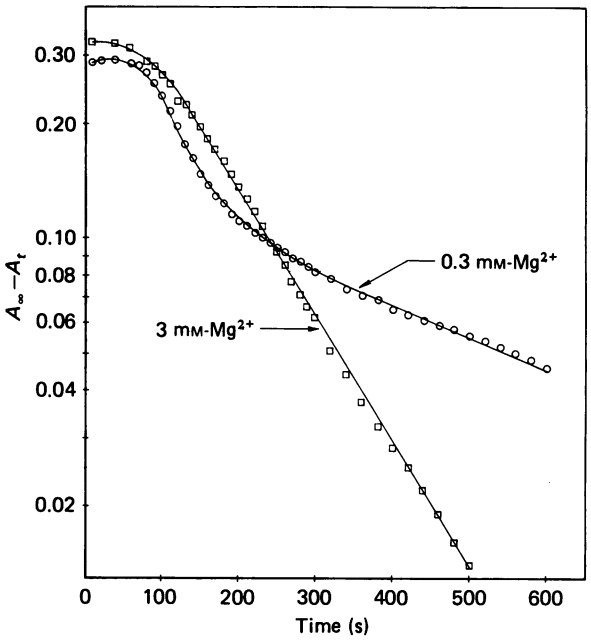

Fig. 3. Effect of Mg^{2+} on the kinetics of assembly

Assembly conditions were 0.1 M-MEMg, pH 6.5 (I0.069), 1 mM-GTP, 1 mM-dithiothreitol, 37 °C and 1.30 mg of microtubule protein/ml. Lines were plotted by using $A = 0.432 \pm 0.005$, $k = 0.00734 \pm 0.00013$ and $A_\infty = 0.464 \pm 0.001$ for 3 mM-Mg^{2+} and by using $A_1 = 0.285 \pm 0.005$, $k_1 = 0.0212 \pm 0.0006$, $A_2 = 0.127 \pm 0.001$, $k_2 = 0.00191 \pm 0.00009$ and $A_\infty = 0.387 \pm 0.002$ for 0.3 mM-Mg^{2+}.

varied with KCl, a plateau was observed from I0.1 to I0.15; thereafter $(A_\infty - A_0)$ decreased as I increased, probably owing to the inhibitory action of KCl. This inhibitory observation is consistent with the findings of Olmsted & Borisy (1973), who showed that KCl was 50% inhibitory at 50 mM.

The type of kinetics observed was not altered by pH in the pH range 6.5–6.9. Since the ionic strength of Pipes buffer varies with pH, it was maintained constant by addition of KCl at the lower pH values. The duration of the fast biphasic step was altered by pH being extended in time at the lower pH (Fig. 2). Comparison of the kinetic parameters calculated according to eqn. (2) reveals alterations in the ratios A_1/A_2 and k_1/k_2. A_1/A_2 is 2-fold higher than, and k_1/k_2 is 70% lower than, the values obtained at pH 6.9. Simulation analyses accomplished with eqn. (2) in the form of $A_\infty - A_t$ demonstrated that increased values of A_1/A_2 and k_1/k_2 both tend to enhance the percentage contribution of the first term to the change in absorbance and to lengthen the duration of the fast biphasic step. For these studies, the primary effect, then, is the enhanced value of A_1/A_2 at pH 6.5. These results seem to disagree with those of Bayley *et al.* (1983), who found monophasic kinetics at pH $\geqslant 7$ and biphasic kinetics at lower pH. This discrepancy may be explained if they did not maintain a constant ionic strength. Moreover, in a later work they observed biphasic kinetics at pH 6.95 (Bayley *et al.*, 1985).

When MEMg buffer, pH 6.5, was varied from 0.1 M (I0.069) to 0.3 M (I0.207), biphasic kinetics were observed until 0.3 M, whereupon monophasic kinetics prevailed. The biphasic character of assembly is more highly emphasized, with the fast step extending to 240 s, when lower-ionic-strength MEMg buffer is compared with either 50 mM- or 100 mM-PEMg.

Effect of Mg^{2+}

Assembly was measured for a range of Mg^{2+} concentrations (0.3 mM–9 mM) in 0.1 M-MEMg, pH 6.5. Mg^{2+} at concentrations greater than, or equal to, 1.2 mM caused a transition from biphasic to monophasic assembly kinetics (Fig. 3). The observed rate constants for fast biphasic and monophasic assembly decreased non-linearly with Mg^{2+}, showing the inhibitory nature of this ion. The maximum extent of reaction, however, was not inhibited until Mg^{2+} reached 6 mM. With a crude extract, Olmsted & Borisy (1973) found complete inhibition at 10 mM. The Mg^{2+}-induced transition to monophasic kinetics occurred at low ionic strength (I0.074) with the $MgSO_4$ contribution to I included in the calculation. Transition at such a low value of I caused us to suspect the effect might be more specific and not related to ionic strength. We therefore varied the ionic strength over the same range by using KCl and found biphasic kinetics, which confirms that this Mg^{2+}-induced transition is not ionic-strength-dependent.

Effect of temperature

Assembly in 0.1 M-MEMg, pH 6.5, was biphasic at temperatures above 30 °C and monophasic below 30 °C. Activation energies obtained by least-squares fitting to

Table 2. Activation energies of microtubule assembly

Assemblies were carried out in indicated buffer containing 1 mM-GTP and 0.5 mM-dithiothreitol.

Conditions of assembly	Temperature (°C)	Activation energy: Reaction 1 (kJ/mol)	Reaction 1 (kcal/mol)	Reaction 2 (kJ/mol)	Reaction 2 (kcal/mol)	Kinetics
0.1 M-MEMg, *I*0.069, pH 6.5*	32–37	113.3	27.1	51.4	12.3	Biphasic
	20–38	120.8	28.9	–	–	Monophasic
0.1 M-PEMg, *I*0.212, pH 6.9†	20–37	116.2	27.8	–	–	Monophasic
0.1 M-MEMg, 3 mM-Mg^{2+}, *I*0.081, pH 6.5‡	26–37	113.7	27.2	–	–	Monophasic
		Average...	27.8			

* Duplicate assemblies at each temperature with 0.7–0.9 mg of microtubule protein/ml.
† Duplicate assemblies at each temperature with microtubule-protein concentration 1 mg/ml.
‡ Microtubule-protein concentration was 1 mg/ml for five, and 1.5 mg/ml for two, determinations in 0.1 M-MEMg, pH 6.5, with the Mg^{2+} concentration raised to 3 mM.

Table 3. Rate constants for microtubule assembly

Assemblies were accomplished in PEMg buffer, pH 6.9, with biphasic results at *I*0.106 and monophasic at *I*0.212. For each entry, rate-constant values were averaged for a single assembly at various times betwen 170 and 1800 s, where MTN is constant in value. The average values represent the results of more than one assembly. In order from top to bottom for *I*0.106 microtubule-protein concentrations were 0.90, 0.99, and 1.09 mg/ml and the number of determinations were eleven, four and three. Likewise, for *I*0.212, the corresponding values were 1.03 and 1.31 mg/ml, with two and five determinations. First-order reverse rate constants were calculated from the relationship derived by Oosawa & Asakura (1975), namely that $C_c = k_-/k_+$; in the absence of contrary evidence, both steps of the biphasic process were assumed to have the same C_c.

	Biphasic kinetics				Monophasic kinetics	
	$10^{-7} \times k_1$ ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	$10^{-6} \times k_2$ ($M^{-1} \cdot s^{-1}$)	k_{-2} (s^{-1})	$10^{-7} \times k_+$ ($M^{-1} \cdot s^{-1}$)	k_- (s^{-1})
	3.80±0.14	76.0±2.9	6.28±0.24	12.6±0.5	2.68±0.15	53.6±0.24
	5.07±0.4	101.4±8.1	6.58±0.52	10.6±2.9	1.55±0.04	31.0±0.09
	2.49±0.03	51.8±0.6	2.70±0.03	5.4±0.1	–	–
Average±S.E.M. ...	3.82±0.72	76.4±14.3	5.19±1.25	9.5±2.1	2.12±0.56	42.3±11.2

the Arrhenius equation were 113.3 kJ (27.1 kcal)/mol and 51.4 kJ (12.3 kcal)/mol for the fast and slow biphasic steps respectively (Table 2). The activation energy of 111.2 kJ (26.6 kcal)/mol measured by Bayley *et al.* (1985) for the fast step agrees with our results, but their value was 89 kJ (21.3 kcal)/mol for the slow step. The activation energy for monophasic kinetics caused by low temperatures (25–28 °C) was 120.8 kJ (28.9 kcal)/mol. Comparison of activation energies indicates that the fast biphasic step is observed at lower temperatures with little contribution from the slow biphasic step. Contribution of both biphasic steps would have yielded an activation energy of about 100.3–104.5 kJ (24–25 kcal)/mol, since for coalescence of two steps the activation energy is a weighted average with the rate constants of the two steps serving as the weights (Laidler & Peterman, 1979).

Temperature was used as a tool to uncover the relationship between monophasic assembly at high *I* and high Mg^{2+}, and biphasic assembly. Temperature was varied from 25 °C to 37 °C for microtubule protein (1 mg/ml) in 0.1 M-PEMg, pH 6.9, and in 0.1 M-MEMg, pH 6.5, with 3 mM-Mg^{2+}. For the PEMg buffer the activation energy was 116.2 kJ (27.8 kcal)/mol, and for the MEMg buffer with 3 mM-Mg^{2+} the activation energy was 113.7 kJ (27.2 kcal)/mol (Table 2). For both of these solutions (0.1 M-PEMg and 0.1 M-MEMg with 3 mM-Mg) yielding monophasic assembly kinetics, the activation energies are identical with the value for the fast step of biphasic kinetics. This finding indicates the same reactant is elongating the tubule at high ionic strength, 3 mM-Mg^{2+}, and during the fast biphasic step at low ionic strength.

Second-order rate constants

On average the observed rate constant for monophasic kinetics is about 2.5 times smaller than the observed rate constant for the fast biphasic step. The observed rate constant is related to the second-order rate constant, where MTN is the microtubule number concentration, by:

$$k_{obs.} = k \cdot \text{MTN}$$

The 2-fold lower rate constant at *I*0.212 could be reflected in the value of k or the value of MTN. To resolve this issue, MTN was calculated from lengths of

microtubules measured from electron micrographs of magnification 8250× using a HIPAD (Houston Instrument Digitizing Pad) in PEMg buffer, pH 6.9; length measurements, made after the absorbance reached A_∞, yielded average values of 10–13 μm. MTN was calculated from the equation given by Farrell & Jordan (1982) after the critical concentration (C_c) was subtracted from the total protein concentration to yield the concentration of polymer. MTN was determined for three assemblies at I0.106, which had an average fast observed rate constant of 0.0285 ± 0.0038 s^{-1} and for two assemblies at I0.212, which had an average observed rate constant of 0.0116 ± 0.0014 s^{-1}. For each assembly the MTN was calculated for five or more data points in the time range 120–1200 s. The average MTN was $6.8(\pm 0.5) \times 10^{-10}$ M at I0.106 and $6.2(\pm 0.4) \times 10^{-10}$ M at I0.212 to give a ratio of 1:1. Lack of variation of MTN values with ionic strength suggests the change in $k_{obs.}$ is to be found in the second-order rate constants. This conclusion is confirmed by the average rate constants presented in Table 3. An average value of 1.8 was computed for the ratio of second-order rate constants of the fast biphasic and the monophasic steps.

The rate constants for the fast biphasic step, given in Table 3, are one order of magnitude larger than those reported by Farrell & Jordan (1982) and Johnson & Borisy (1977). Two factors might account for this difference. Our observation of longer microtubules would result in a decrease in the microtubule number concentration, and those authors used a lower temperature for assembly. Gal *et al.* (1986), who also observed biphasic kinetics, reported microtubule lengths consistent with those reported here.

Association constant for microtubule elongation

When C_c values were measured for the three buffer conditions, no significant differences were found: C_c in mg/ml was 0.12 ± 0.08 for 50 mM-PEMg, pH 6.9, 0.19 ± 0.03 for 100 mM-PEMg, pH 6.9, and 0.15 for 100 mM-MEMg, pH 6.5. Each individual value was obtained by linear regression and then averaged with others to give the stated values.

Using a tubulin dimer M_r of 100000 (Ponstingl *et al.*, 1981; Valenzuela *et al.*, 1981) and the C_c, we calculated an association constant of 5×10^5 M^{-1} for 0.1 M-PEMg, pH 6.9, in agreement with the values reported by Johnson & Borisy (1977).

MTN

The possibility of changes in MTN accounting for biphasic assembly was investigated. Microtubule lengths were measured on samples from 60 to 1200 s. For this analysis, the microtubule molar concentration, [MT], at any time was calculated from eqn. (3).

$$[\mathrm{MT}] = \frac{A_t - A_0}{A_\infty - A_0} \times \frac{C_0 - C_c}{100000} \qquad (3)$$

where C_0 is the total microtubule-protein concentration and C_c is as already defined. No change in number concentration was observed from 120 to 1200 s for 50 mM-PEMg, pH 6.9, and 100 mM-PEMg, pH 6.9. In making this assessment, 822 and 548 microtubules were measured for I0.106 and I0.212 respectively. The decision of invariant MTN was based on a one-way analysis of variance, using a robust value of F by Levene (1960) at the 5% level of significance. The F value for the time range including 90 s is 13% higher than the statistical F at 5% significance. When 2.5% is the level of significance, MTN is invariant with time from 90 to 1200 s in 50 mM-PEMg, pH 6.9.

DISCUSSION

We have demonstrated that the type of assembly kinetics displayed by microtubule protein is dependent on I but not the pH. Lower pH (6.5 rather than 6.9) does, however, exaggerate biphasic kinetics by extending the duration of the fast step. Observations of biphasic kinetics at low I and monophasic kinetics at high I brings into agreement the data of Barton & Riazi (1980) and Johnson & Borisy (1977).

Analysis of available evidence leads to the conclusion that both biphasic assembly steps involve elongation rather than nucleation of microtubules. The basis of this conclusion is as follows. The constancy of the MTN value signifies that the non-linearity of the first-order plot of biphasic assembly is not attributable to changes in the value of MTN. The data for each step of biphasic assembly independently yield pseudo-first-order-kinetics plots which confirms the adequacy of eqn. (2) for describing biphasic assembly. The data fit to eqns. (1) and (2) were taken after a 10% change in absorbance had occurred in order to eliminate inclusion of any part of the nucleation stage (Purich & Kristofferson, 1984). Also, by electron microscopy, fully formed microtubules were observed as early as 60 s after initiation of assembly by temperature jump. By 90 s the average length was 2 μm, and lengths continued to grow to 10 μm by 1200 s. These events are consistent with those observed by Erickson (1974), who reported predominantly short microtubules in the early growth times of 1–2 min.

Biphasic kinetics, described by a sum of exponentials, implies two parallel reactions with different reactants yielding the same product (Moore & Pearson, 1981). For microtubule growth, the following scheme represents the biphasic results. The nucleus of each reaction represents the microtubule newly emerged from the nucleation process:

$$\text{Nucleus 1} + \text{reactant 1} \xrightarrow{k_{fast}} \text{microtubule} \quad (F)$$

$$\text{Nucleus 2} + \text{reactant 2} \xrightarrow{k_{slow}} \text{microtubule} \quad (S)$$

Eqn. (2) was used in the form:

$$A_\infty - A_t = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

to assess the contribution of each exponential term to the absorbance difference as a function of time. When rate constants and amplitudes for a typical biphasic result were applied to the rearranged eqn. (2), the exponential term due to the fast step accounted for 51% of the absorbance difference at $t = 10$ s and decreased to 0% contribution by 470 s, confirming the parallel or simultaneous nature of these growth reactions.

At this point the nature of reactants for reactions (F) and (S) are unknown. Some possibilities for these reactants are tubulin dimer, tubulin dimer–MAP complex and tubulin ring fragments rich in MAPs. On the basis of the addition of tubulin dimer to seed microtubules, Johnson & Borisy (1977) favour dimer addition for the observed monophasic step. It is not possible, however, for tubulin dimer alone to account for biphasic assembly kinetics, because dimer cannot serve as the reactant in

both reactions (F) and (S). However, either dimer complexed with MAPs and pure dimer or dimer complexed with two different MAPs could serve as reactants 1 and 2. Alternatively, an aggregate composed only of tubulin could serve as one reactant and dimer as the other. Such an oligomer of tubulin does not seem likely, however, because it should also form from tubulin purified by phosphocellulose, and there is no evidence of biphasic kinetics on the part of such highly purified tubulin. Moreover, observation of the existence of oligomers has been associated with the presence of MAPs or other cationic agents.

There is some experimental evidence for a complex of tubulin and MAPs serving as reactant 1. Kumar (1981) observed biphasic assembly of phosphocellulose-purified tubulin only in the presence of MAPs. Bayley *et al.* (1985) demonstrated, by SDS/polyacrylamide-gel-electrophoretic analysis, the rapid loss of MAPs from the supernatant when microtubules were sedimented from solution at various times of assembly. In the same work, Bayley's group (Bayley *et al.*, 1985) also reported ring opening and dissociation to oligomeric fragments to have the same observed rate constant and activation energy as the fast biphasic step. According to Pantaloni *et al.* (1981), 90% of the tubule formed in the first few minutes came from ring fragments. Mandelkow *et al.* (1980), who showed disruption of rings into smaller protofilament fragments but not into dimers, favour a model where these fragments are used solely for nucleation, with tubulin dimer the elongating agent (Renner *et al.*, 1983). However, they are unable to distinguish between short protofilament fragments and tubulin dimer by the time-resolved X-ray-scattering experiments (Bordas *et al.*, 1983). According to Burns & Islam (1984), a simple pseudo-first-order kinetic process takes place at 20 μM-GTP, but at 1 mM-GTP, two pseudo-first-order processes are the assembly mode. Those authors attribute biphasic assembly to nucleotide binding to oligomer and its incorporation into microtubules at the higher GTP concentration. We have confirmed that monophasic kinetics is observed at 25 μM-GTP. When these results are taken together, it appears that the protofilament fragments rich in MAPs are candidates for reactant 1 of reaction (F).

To serve as a reactant, an oligomeric form must be capable of existing at 37 °C and I0.2. Marcum & Borisy (1978) demonstrated the existence of an 18S oligomer at I0.2 and 5 °C; subsequently, Bayley *et al.* (1982) confirmed that high I favours the 18S form. According to Mandelkow *et al.* (1980), an oligomeric fragment does exist during the early stages of assembly. Previously, Barton & Riazi (1982) reported the existence of oligomers at 30 °C and I0.082. In the present investigation, oligomeric forms were found to exist at 10 °C and 30 °C for I0.1 and 0.2. The analyses were accomplished by h.p.l.c. gel filtration on a column (300 mm × 7.5 mm) of BIO-SIL TSK 250 in the absence of nucleotide. For all running conditions, there were two protein boundaries corresponding to an oligomer exiting just before thyroglobulin (M_r 670000) and a dimer of M_r 107000.

Our ionic-strength results are consistent with either a tubulin dimer–MAP complex or tubulin oligomer–MAP complex serving as reactant 1. At low I, there could exist two pools of tubulin, one rich in MAPs and a second deficient in MAPs. At high I, electrostatic interaction between MAPs and tubulin could be altered allowing a redistribution of MAPs such that there exists only one type of tubulin, interacting less effectively with MAPs. This rearranged complex at high I would add with a second-order rate constant that is 2-fold lower than that for the MAP-rich tubulin and 3-fold faster than that for the MAP-deficient tubulin. Alternatively, the two pools could consist of tubulin complexed with two different MAPs with distinguishable strengths of interaction. At high I, one of these complexes could be less stable.

Regardless of the nature of reactants, the equivalence of activation energies for the fast biphasic step at low I and the monophasic step at high I, argues for the same type of reactant for both steps.

Mg^{2+} at concentrations above 1 mM caused a transition from biphasic to monophasic assembly kinetics. On the basis of activation energies, the tubulin reactant at high Mg^{2+} is the same as reactant 1 for the fast biphasic step. One possible explanation of this observation is the interaction of Mg^{2+} with tubulin dimer to form a species similar to the fast biphasic reactant. This Mg^{2+}-induced tubulin species could be an oligomeric form that is protofilament in character. Support for this suggestion is taken from Frigon & Timasheff (1975*a*), who demonstrated Mg^{2+}-induced oligomer formation. They postulated three possibilities for the action of Mg^{2+}: (i) to serve as a direct linkage between dimers, (ii) to mask repulsive electrostatic interactions or (iii) to induce a conformational change (Frigon & Timasheff, 1975*b*). Thus, two kinds of oligomers might exist: one due to MAPs and the other due to Mg^{2+}, and both would have to add to growing microtubules similarly. A second possibility is an Mg^{2+}-caused redistribution of MAPs such that each tubulin species added in coincidence with MAPs. This redistribution, not caused by ionic strength, would require a specific interaction by Mg^{2+} such as displacement or competition with MAPs.

The proposed model accounts for all of the presented data, but does not delineate the nature of reactants 1 and 2. The identity of these reactants, only inferred from these studies, has yet to be determined. This model is similar to that of Bayley *et al.* (1985), which is slightly more complex.

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