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Effects of GDP on microtubules at steady state

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We have re-examined the effect of varying GDP concentrations on the kinetics of GTP-induced assembly of microtubules from microtubule protein, and on the elongation of pre-existing microtubules subjected to a temperature jump relaxation from 21.5 to 37 ° C. The assembly kinetics follow a simple model for assembly which involves a fast equilibrium of tubulin-GTP and tubulin-GDP coupled to the elongation process due to tubulin-GTP. The initial rate of the relaxation process is found to be dependent upon the GTP/GDP ratio, in confirmation of the results of Engelborghs and Van Houtte (Biophys. Chem. 14 (1981) 195). As an alternative to the interpretation previously advanced by them, involving modification of the reactivity of microtubule ENDs by GDP, we show that this result is consistent with the above model with one reasonable modification, namely, that the ratio of the affinities of tubulin for GTP and GDP should vary with temperature. The analysis shows that a decrease in this ratio of approx. 2-fold at 37 ° C accounts for the observed effects. We conclude that more complex mechanisms involving consideration of modification of the reactivity of microtubule ENDs by GDP are not required to explain these results. This finding has important implications for current models of GDP-induced microtubule disassembly.

1. Introduction

GDP is known to be a potent inhibitor of microtubule assembly, it reduces the rate and amplitude of assembly of GTP-induced microtubule assembly from microtubule protein or from tubulin dimer, and added GDP promotes disassembly from the steady state [1–8]. The fact that the same extent of assembly is reached whether GDP is added during elongation (but after nucleation) or at steady state [4,8] strongly suggests the operation of relatively simple equilibria. This possibility led us to reassess the experimental evidence concerning the effect of GDP upon microtubules.

In considering the effect of GDP on microtubule elongation and steady-state GTP hydrolysis, it had previously been postulated that tubulin-GTP cannot add to a microtubule end with a tubulin-GDP in the terminal position [8,9]. This concept has been used in extensive modelling of the formation of a GTP cap as a regulator of microtubule stability [10–12]. We recently showed, by computer simulation [13], that the effect of the nucleotide exchange reaction between tubulin-GTP and tubulin-GDP coupled to the elongation reaction of tubulin-GTP is able to account for many of the effects of GDP on assembly in the presence of GTP.

This coupled mechanism, suggested by Jameson and Caplow [6] was evaluated in detail by

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Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; MAPs, microtubule-associated proteins; tubulin-GTP (Tu-GTP), tubulin with GTP bound; tubulin GDP (Tu-GDP), tubulin with GDP bound; MT-protein, cold-dissociated microtubule protein; Mes, 2-(*N*-morpholino)-ethanesulphonic acid; S^6-GTP, 2-amino-6-mercapto-9-ribofuranosylpurine 5′-triphosphate.

Engelborghs and Van Houtte [7] for the relaxation process involving small changes in assembly/disassembly at the steady state due to a temperature jump. Because of the nature of the polymerisation kinetics, the results are equally applicable to the 'full' assembly reaction.

These authors [7] also sought an experimental test of whether the nucleotide composition of the microtubule ends influenced the elongation kinetics. Microtubules initially at steady state in the presence of various GDP/GTP mixtures at 25°C were subjected to a rapid temperature jump to 35°C and the slope of the first portion of the assembly curve monitored to give an 'initial rate'. The dependence of this rate on the [GTP]/[GDP] ratio was used to exclude the simple coupled mechanism in favour of more complex models involving modification of the reactivity of the microtubule end due to the presence of bound GDP.

We have reinvestigated experimentally the 'full' assembly of microtubules from microtubule protein in the presence of GTP and GDP and analysed the data to obtain a value for a, the ratio of nucleotide association constants for tubulin-GTP and tubulin-GDP at 37°C. We repeated the (relaxation) experiments of Engelborghs and Van Houtte [7] and confirmed that the initial rate is indeed dependent upon the GDP/GTP ratio. However, we propose a relatively simple explanation for this effect which does not involve the nucleotide composition of the ends, but depends upon a small difference in the relative affinities for tubulin GTP and GDP at the two temperatures. It is found that a decrease of the ratio of association constants K_{GTP}/K_{GDP} of less than 2-fold between 21.5 and 37°C is sufficient to explain the result.

Thus, the simple mechanism of a rapid exchange between tubulin-GTP and tubulin-GDP is able to account for the effects of GDP on the rate and amplitude of GTP-induced microtubule assembly. This mechanism also accounts for the initial rate of assembly in a subsequent relaxation experiment, provided allowance is made for the appropriate temperature effects on nucleotide affinities.

2. Materials and methods

MT-protein was prepared from bovine brain by two cycles of assembly and disassembly in Mes buffer, pH 6.5, in the presence of glycerol by a modification of the method of Shelanski et al. [14] (see ref. 15). Before each experiment the stored MT-protein solution was taken through a further cycle of assembly, resuspended in MEM100 buffer (100 mM Mes, 0.5 mM $MgCl_2$, 0.1 mM EGTA, pH 6.50) and clarified by centrifugation at 4°C. Residual nucleotide was removed by passage through a Sephadex G-25 column.

The effect of GDP on the rate and amplitude of the full assembly from cold MT-protein solution was studied in a Cary 118 spectrophotometer by monitoring turbidity at 330 nm. The protein solution at 0.92 mg/ml in MEM100 buffer was made 0.4 mM in GTP and placed in a thermostatted 10 mm cuvette at 7.5°C. The temperature was then rapidly raised to 37°C and a small aliquot of concentrated GDP was added after the turbidity had started to increase (i.e., at 120 s). The final concentration of GDP was in the range 0–4 mM. The progress curve was recorded by on-line analogue-to-digital conversion into a PDP 11/23 computer, with data averaging to give an effective acquisition time of 4 s per point, and an overall scan time of 800–1200 s.

The effect of GDP on the rate and kinetics of the relaxation process following a temperature jump from 21.5 to 37°C was studied in a similar way (cf. ref. 7). A protein solution at 2.62 (or 2.56) mg/ml in MEM100 buffer was made 0.4 mM in GTP and placed in a 10 mm cuvette at 7.5°C. The temperature was then raised to 37°C and the assembly process was allowed to proceed to completion without the addition of GDP. At this point, a small aliquot of concentrated GDP was added (final concentration in the range 0–3.2 mM) and simultaneously the temperature was lowered to 21.5°C. The decrease in turbidity brought about by these changes was allowed to proceed to completion and the new turbidity level was noted. Finally, the temperature was again increased to 37°C and the kinetics of the relaxation process were digitized as described above. (This protocol enables the steady state in the presence of GDP at

21.5 °C to be achieved more rapidly than by direct assembly).

Because the analysis to be presented here depends upon knowledge of the GTP/GDP ratio we investigated the contribution of non-specific GTPase activity to the hydrolysis of GTP. MT-protein (1 mg/ml Sephadex G-25 treated; 0.4 mM GTP) was assembled at 37 °C and samples were taken at 15-min intervals for nucleotide analysis by HPLC. The amount of GTP hydrolysed was 15% (15 min), 29% (30 min), 44% (45 min) and 53% (60 min). Therefore, in the relaxation experiments (which were generally started within 15–20 min of the start of the initial assembly) we would expect that less than 20% of the GTP would have been hydrolysed. In order to simplify the calculations we have assumed that no GTP is hydrolysed. However, the effect of this level of hydrolysis is discussed at various points in the text.

For both assembly and relaxation experiments most of the assembly curve could be fitted with a single exponential function such that the time-dependent signal, A_t, is given by:

$$A_t = A_0 - A \cdot \exp(-k_{obs} t) \quad (1)$$

or by

$$A_t = A_0 - A_r \cdot \exp(-t/\tau) \quad (2)$$

in the case of the relaxation experiment.

Non-linear least-squares analysis provides values for the observed rate constant, k_{obs} (or relaxation time, τ) and the reaction amplitude, A (or A_r for the relaxation experiment).

In the case of the full assembly curves the initial portion shows some acceleration (see fig. 1b) which is probably due to a small amount of residual nucleation. In the computer analysis we progressively drop increasing numbers of points from the front of the curve until the parameters returned by the program remain constant. In most cases this procedure resulted in a good fit to at least 80% of the curve. In the case of the relaxation curves we obtained good single-exponential fits over the full time course (fig. 1c).

3. Theory

3.1. Full assembly (no GDP)

For the reaction

$$\mathrm{TuGTP} + \mathrm{END} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{END}$$

the observed rate constant is given by: $k_{obs} = k_1[\mathrm{END}]$. For the full assembly process at 37 °C the reaction amplitude is given by:

$$A = FC_t - FC_c = F(C_t - C_c)$$

where F is the 'specific turbidity' (i.e., apparent absorbance/mg per ml assembled tubulin), C_t the total tubulin concentration and C_c the critical concentration at 37 °C.

3.2. Relaxation (no GDP)

In the relaxation experiments, the relaxation time is given by $1/\tau = k_1[\mathrm{END}]$ and for this relaxation process, the amplitude for a temperature jump from 21.5 to 37 °C is given by:

$$A_r = (FC_t - FC_c) - (FC_t - FC_c') = F(C_c' - C_c)$$

where C_c' is the critical concentration at 21.5 °C.

3.3. Full assembly (plus GDP)

Now consider the same reaction in the presence of added GDP, i.e.

$$\mathrm{TuGDP} + \mathrm{GTP} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \mathrm{GDP} + \mathrm{TuGTP} + \mathrm{END} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{END}$$

Under pseudo first-order conditions with respect to both ligands ($[\mathrm{GTP}]_t \gg C_t$ and $[\mathrm{GDP}]_t \gg C_t$; where the subscript on the nucleotide concentration indicates that it signifies total concentration) and assuming that the nucleotide exchange reaction is fast compared with the elongation step, it is readily shown that:

$$k_{obs} = \frac{k_1[\mathrm{END}]k_4[\mathrm{GTP}]_t}{k_3[\mathrm{GDP}]_t + k_4[\mathrm{GTP}]_t} \quad (3)$$

Letting $a = K_{GTP}/K_{GDP} = k_4/k_3$ (where the K terms are the association constants for nucleotide binding) and $R = [GTP]_t/[GDP]_t$, it is readily shown that:

$$[TuGTP]/[TuGDP] = x = aR$$

Substituting in eq. 3

$$k_{obs} = k_1[END]x/(1+x) \quad (4)$$

Note that a (and thus x) is calculated using K_{GTP} and K_{GDP}, values of the association constant that apply at 37°C.

The amplitude for the full assembly experiment is given by:

$$\begin{aligned} A &= FC_t - F[TuGTP] - F[TuGDP] \\ &= FC_t - FC_c - F[TuGTP]/x \\ &= FC_t - FC_c(1+x)/x \end{aligned} \quad (5)$$

It may be noted that the initial rate, k_{init}, for the full assembly process is given by:

$$\begin{aligned} k_{init} &= k_{obs}A \\ &= [k_1[END]x/(1+x)] \\ &\quad \times [FC_t - FC_c(1+x)/x] \end{aligned} \quad (6)$$

When R decreases (i.e., in the presence of added GDP), $x/(1+x)$ decreases and $(1+x)/x$ correspondingly increases. Therefore, the initial rate in this system should be very strongly dependent upon the concentration of added GDP.

3.4. Relaxation (plus GDP)

The relaxation time for the temperature jump experiment is defined by $1/\tau = k_1[END]x/(1+x)$ (as above and see ref. 7) and the amplitude for the relaxation experiment is given by:

$$\begin{aligned} A_r &= (FC_t - FC_c(1+x)/x) \\ &\quad - (FC_t - C_c'(1+x')/x') \\ &= FC_c'(1+x')/x' - FC_c(1+x)/x \end{aligned} \quad (7)$$

with $x' = a'R = K'_{GTP}[GTP]_t/K'_{GDP}[GDP]_t$, where a' (and thus x') is calculated using K'_{GTP} and K'_{GDP}, the values of the association constants that apply at 21.5°C.

If $a = a'$ (i.e., a is independent of temperature) then eq. 7 reduces to:

$$A_r = F(C_c' - C_c)(1+x)/x \quad (8)$$

The initial rate, k_{init} (as defined above), for the relaxation experiment is then given by:

$$\begin{aligned} k_{init} &= A_r/\tau \\ &= [k_1[END]x/(1+x)] \\ &\quad \times [F(C_c' - C_c)(1+x)/x] \\ &= k_1[END]F(C_c' - C_c) \end{aligned} \quad (9)$$

i.e., the initial rate for the relaxation experiment is predicted to be independent of $[GTP]_t/[GDP]_t$ only if the ratio of the association constants (K_{GTP}/K_{GDP}) does not vary with temperature.

When a does not equal a' then k_{init} is given by:

$$\begin{aligned} k_{init} &= [k_1[END]x/(1+x)] \\ &\quad \times [FC_c'(1+x')/x' - FC_c(1+x)/x] \end{aligned} \quad (10)$$

and its dependence upon $[GTP]_t/[GDP]_t$ will be determined by the difference between a and a'.

4. Results

4.1. Full assembly data

Fig. 1a shows some of the experimental data obtained in the study of the full assembly process. It should be noted that with [GTP] = 0.4 mM the assembly curve is accurately fitted with a single exponential function over most of the time course (see section 2), for all GDP concentrations studied. In the absence of added GDP the observed rate constant, k_{obs}, is found to be 0.0122 (±0.001) s^{-1} and the amplitude (at 0.92 mg/ml) is 0.135 (±0.005). Previous studies of the assembly process performed at higher [GTP] [16] showed biphasic assembly curves with observed rate constants of 0.0317 (±0.006) and 0.0036 (±0.0007) s^{-1} and an overall reaction amplitude (at 1.0 mg/ml) of 0.157 (±0.021). The disappearance of the slow phase at lower GTP concentrations has also been demonstrated by Burns and Islam [17].

To study the effect of added GDP, the point of

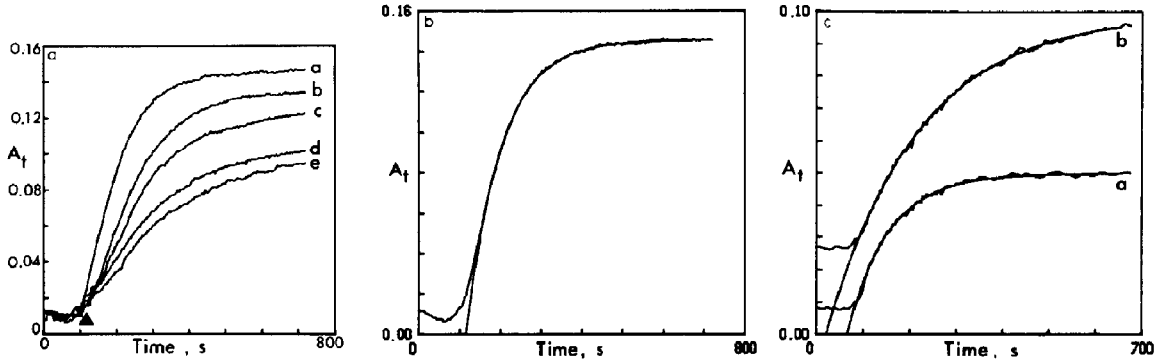

Fig. 1. (a) Full assembly curves for the assembly of MT-protein in the presence of 0.4 mM GTP and different GDP concentrations (MEM100, 37°C). GDP concentrations were 0 (a), 0.833 (b), 1.25 (c), 1.875 (d) and 2.083 mM (e). The black triangle indicates the point at which GDP was added. (b) Full assembly curve and computed fit for no added GDP (curve a in panel a). (c) Relaxation curves with computed fits for 0 (a) and 2.4 mM (b) added GDP.

addition (120 s) is after the turbidity has started to increase; this is 'post-nucleation' and hence the assemblies are performed at constant microtubule END concentration. As expected from eqs. 4 and 5, the observed rate constant and amplitude both decrease significantly as the GDP concentration is increased. Typical values for the observed rate constant, the total reaction amplitude and the calculated initial rate ($= k_{obs}A$) are listed in table 1. Initial rates (i.e., slopes) are not easily measured in the conventional way because the addition of GDP perturbs the initial portion of the curve. Note that the initial rate is reduced by a factor of 18 at the highest GDP concentration used, in agreement with the strong dependence on GDP concentration predicted by eq. 6.

In principle, these data can now be analysed

Table 1

Effect of GDP on the observed rate constants, k_{obs}, amplitudes, A, and initial rates, k_{init}, for 'full' assembly of MT-protein (0. 92 mg/ml; 0.4 mM GTP) in MEM100 buffer at 37°C

[GDP] (mM)	$k_{obs}(\times 10^3)$	A	$k_{init}(\times 10^3)$ [a]
0.000	12.60	0.142	1.789
0.208	10.82	0.133	1.439
0.416	10.35	0.132	1.366
0.833	8.10	0.125	1.012
1.666	6.70	0.103	0.690
2.083	4.55	0.088	0.400
4.166	2.82	0.038	0.107

[a] Values of k_{init} were calculated as $k_{init} = k_{obs}A$ (see text).

using eq. 4 for the rate data and eq. 5 for the amplitude data. However, these plots require an accurate value for a, the ratio K_{GTP}/K_{GDP} where the K values are those that apply at 37°C. Zeeberg and Caplow [18] measured the association constants for GDP and GTP binding to tubulin dimer prepared by Sepharose 6B chromatography using the Hummel-Dryer technique and obtained values of $K_{GTP} = 4.54 \times 10^7$ M^{-1} and $K_{GDP} = 1.64 \times 10^7$ M^{-1} at 22°C, giving $a = 2.8$. Fishback and Yarbrough [19] measured the binding of GTP and GDP to phosphocellulose-purified tubulin dimer at 21°C by competition with S^6-GTP and found $K_{GTP} = 5.88 \times 10^7$ M^{-1} and $K_{GDP} = 1.21 \times 10^7$ M^{-1} in 25% glycerol solution, giving $a = 4.86$. Finally, Carlier and Pantaloni [2], using phosphocellulose-purified tubulin dimer, in a buffer containing 25% glycerol, deduced from kinetic experiments that $a = K_{GTP}/K_{GDP} = 6$ at 37°C.

We have plotted the amplitude and rate data according to eqs. 5 and 4 respectively, for a range of a values in order to study the effect of varying a on the desired parameters. These results (table 2) also include the analysis for the case where $a = 2.8$, the value obtained by Zeeberg and Caplow [18] for similar buffer conditions to ours.

Table 2a shows that the calculated value of the specific turbidity, F, is rather insensitive to the value of a chosen, and similarly the values for C_c appear quite reasonable for a values in the range

Table 2

Analysis of experimental amplitude and rate data for the 'full' assembly of MT-protein (0.92 mg/ml; 0.4 mM GTP) in MEM100 at 37 °C, according to eqs. 4 and 5, calculated as a function of a ($= K_{GTP}/K_{GDP}$)

(a) Amplitude data (eq. 5)

a	Intercept	$F = \text{Int}/C_t$	Slope	C_c = slope/intercept
2.8	0.174	0.189	−0.0305	0.161
1.0	0.154	0.167	−0.0109	0.065
1.5	0.159	0.173	−0.0163	0.094
2.0	0.165	0.179	−0.0217	0.121
2.2	0.167	0.182	−0.0232	0.130
2.5	0.170	0.185	−0.0258	0.139
3.0	0.178	0.194	−0.0329	0.169
3.5	0.182	0.197	−0.0381	0.193
4.0	0.187	0.203	−0.0436	0.215

(b) Rate data (eq. 4)

a	Intercept	Slope ($\times 10^3$)
2.8	−0.817	13.99
1.0	1.953	15.32
1.5	1.087	14.25
2.0	0.311	13.93
2.2	0.0	13.95
2.5	−0.406	13.96
3.0	−1.085	14.13
3.5	−1.734	14.29
4.0	−2.360	14.57

1.5–3.0. Fig. 2 shows the plot according to eq. 5 for the cases where a is equal to 2.2 or 2.8. Table 2b shows that the analysis of the kinetic data is somewhat more diagnostic. The intercept of the plot should be zero (see eq. 4). The calculated error for the intercept (from linear regression) is ±0.4 and, therefore, values of a between 1.9 and 2.5 are probably acceptable (see table 1). Fig. 3 shows plots of the data for the cases where a equals 2.2 and 2.8.

The value of a predicted by analysis of the kinetic data (i.e., the value which gives a zero intercept for the plot of eq. 4) is 2.2. With this value for a ($= K_{GTP}/K_{GDP}$) at 37 °C we see from table 2 that $F = 0.182$/mg per ml assembled tubulin and $C_c = 0.130$ mg/ml. These values are typical of those previously found for this system (ref. 15 and Martin and Bayley, unpublished observations).

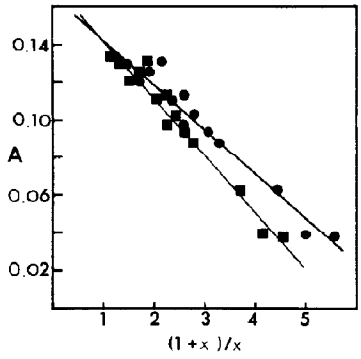

Fig. 2. Analysis of amplitude data from the full assembly experiment. Plot of observed amplitude vs. $(1+x)/x$ using $a = 2.2$ (●) or $a = 2.8$ (■). See text for full details.

4.2. Relaxation data

We first consider the rate data. A plot of $1/\tau$ vs. $x/(1+x)$ using $a = 2.2$ is shown in fig. 4. The intercept is 0.82 (±0.86) and the slope is 13.5 $(\pm 1.4) \times 10^{-3}$ s^{-1}. Considering the magnitude of the errors associated with the determination of τ values from these small amplitude curves these values are in good agreement with the corresponding values of 0.0 and 13.95×10^{-3} s^{-1} obtained for the full assembly experiment (see table 2). Values for reciprocal relaxation times, $1/\tau$, relaxation amplitudes, A_r, and calculated initial rates are given in table 3. The value of the calculated initial rate appears to depend upon the GTP/GDP ratio, being lower at high GDP concentrations as predicted by eq. 10. The variation in initial rate is very similar to that reported by Engelborghs and Van Houtte [7]. This result suggests that a and a' are not in fact equal but the data points do show considerable scatter.

The fact that a does not equal a' can be demonstrated graphically; if the value of a ($= K_{GTP}/K_{GDP}$) was independent of temperature, analysing the amplitude data from the relaxation experiment using eq. 8 (i.e., plotting relaxation amplitude, A_r, vs. $(1+x)/x$) would yield a linear plot with slope $F(C_c' - C_c)$ and intercept zero. This plot is shown in fig. 5. The intercept is clearly not zero, confirming that a cannot be equal to a'.

The deviation from the expected behaviour cannot be explained in terms of the hydrolysis of

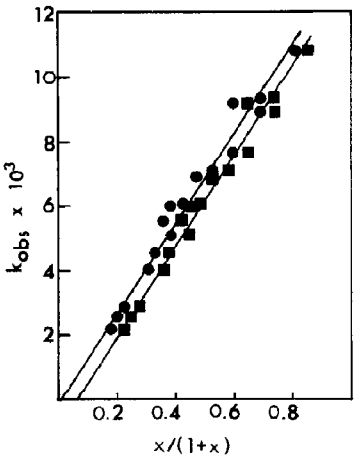

Fig. 3. Analysis of rate data from the full assembly experiment. Plot of observed rate constant vs. $x/(1+x)$ using $a = 2.2$ (●) or $a = 2.8$ (■). See text for full details.

GTP by non-specific GTPases (see section 2). If we assume that 25% of the GTP had been hydrolysed at the start of the relaxation experiment, then the slope of the plot in fig. 5 would be reduced from 0.0141 to 0.0109 whilst the intercept would only change from 0.0272 to 0.0262.

We can now use the amplitude data to estimate a value for a', the ratio of the association constants at 21.5°C. C_c' can be obtained from the relaxation experiment performed in the absence of added GDP. The change in absorbance, from several repeated experiments is 0.042 ($\pm$0.003);

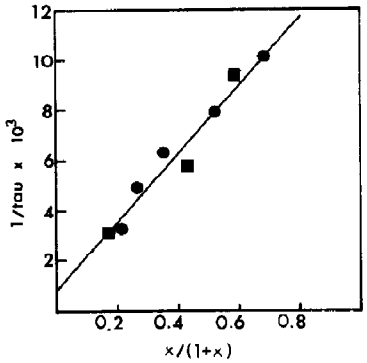

Fig. 4. Analysis of rate data from the relaxation experiment. Plot of observed reciprocal relaxation time vs. $x/(1+x)$ using $a = 2.2$. The two types of symbols indicate that the data were from two separate experiments.

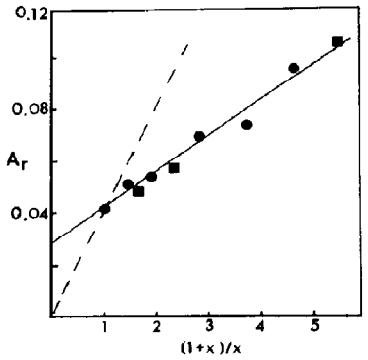

Fig. 5. Analysis of amplitude data from the relaxation experiment. Plot of observed amplitude vs. $(1+x)/x$ using $a = 2.2$ The dashed line is the line expected if the ratio of the association constants is independent of temperature. See text for full details.

with $F = 0.182$ this corresponds to a difference in critical concentration of 0.230 mg/ml between C_c and C_c'. Since $C_c = 0.130$ (see table 2 and above discussion) then $C_c' = 0.230 + 0.130 = 0.360$ mg/ml. Therefore, the slope of the plot according to eq. 8 should be $F(C_c' - C_c) = 0.182 \times 0.230 = 0.042$. This theoretical line is shown as the dashed line in fig. 4; the fact that it is very different from the experimental line confirms that the difference between a and a' must be quite significant.

From eq. 7 since F, C_c, C_c' and x are known from the above considerations, then x' and thus a' can be calculated. The results of this calculation are shown in table 4; this table also contains values of a' calculated for the situation where

Table 3

Effect of [GDP] on the observed reciprocal relaxation times, $1/\tau$, relaxation amplitudes, A_r, and initial rates, k_{init} for a temperature jump from 21.5 to 37°C, for microtubules in the presence of 0.4 mM GTP in MEM100 buffer

[GDP] (mM)	$1/\tau$ ($\times 10^3$)	A_r	k_{init} ($\times 10^3$)
4.0	3.2	0.106	0.339
3.2	3.25	0.096	0.312
2.4	4.95	0.074	0.316
1.6	6.30	0.070	0.441
1.2	5.85	0.059	0.345
0.8	7.90	0.054	0.427
0.6	9.70	0.048	0.475
0.4	10.60	0.052	0.528

Table 4

Values of a' ($= K'_{GTP}/K'_{GDP}$) calculated for relaxation data from eq. 7 for three values of a ($= K_{GTP}/K_{GDP}$) 1.5, 2.2 or 3.0 and using values of F, C_c and C'_c derived as described in the text (prime denotes values at 21.5°C)

[GTP]/[GDP]	a'		
	$a = 1.5$	$a = 2.2$	$a = 3.0$
0.10	3.38	3.82	4.31
0.125	3.31	3.74	4.22
0.250	3.32	3.68	4.16
0.333	3.53	3.98	4.49
0.500	3.46	3.89	4.40
0.666	3.93	4.23	4.98
1.000	2.80	3.13	3.55
Average	3.42	3.81	4.34

$a = 1.5$ (in this case, $F = 0.173$, $C_c = 0.094$ and $C'_c = 0.337$) or $a = 3.0$ (in this case $F = 0.194$, $C_c = 0.169$ and $C'_c = 0.385$). These values were included because they represent the range in the value of a. The average value calculated for a' is 3.42 (for $a = 1.5$), 3.81 (for $a = 2.2$) and 4.34 (for $a = 3.0$). We therefore determine that the ratio of association constants at 21.5°C is 3.81, but that any value in the range 3.42–4.34 is consistent with the data.

The calculated value of a' does, of course, depend on the exact value of the ratio, R. If $a = 2.2$, then the calculated value of a' is 3.81 for no hydrolysis of GTP, 4.24 for 25% and 4.75 for 50% hydrolysis. Thus, the general conclusion that a is not equal to a' is not affected by the hydrolysis of GTP by non-specific GTPases.

5. Discussion

The experimental data presented in fig. 1 and table 1 clearly show that the presence of GDP reduces both the rate and amplitude of assembly of GTP-induced microtubule assembly from microtubule protein under the buffer conditions employed in this study. The calculated value of the initial rate is decreased by a factor of approx. 18 at the highest GDP concentration used. These results are in agreement with many previous studies on the effect of GDP [1–8].

Analysis of these data using eqs. 4 and 5 requires knowledge of the value of a ($= K_{GTP}/K_{GDP}$) at 37°C. Because of the uncertainty in the reported values and the experimental difficulty in obtaining a at 37°C, we analysed our data for a range of values of a distributed about 2.8. Analysis of the amplitude data showed that values of a in the range 1.5–3.0 were reasonable, giving values of F and C_c (eq. 5) in agreement with those derived by other measurements. Analysis of the kinetic data (eq. 4) narrows the acceptable range for a to 1.9–2.5, based on the fact that the intercept should be zero. This is given by $a = 2.2$, from which we derive values for the other parameters: $F = 0.182$, $C_c = 0.130$ and $k_1[\mathrm{END}] = 13.95 \times 10^{-3}\ \mathrm{s}^{-1}$. This analysis is outlined in full in table 2 and representative plots are shown in figs. 2 and 3.

The analysis of the reciprocal relaxation time data is straightforward. Using $a = 2.2$ we have plotted $1/\tau$ vs. $x/(1+x)$ in fig. 4. The value of $k_1[\mathrm{END}]$ obtained from this plot agrees well with that derived for the analysis of the full assembly rate data, as expected, and the intercept is close to zero.

The analysis of the relaxation amplitude data hinges on the question of whether or not the ratio of the association constants is temperature dependent. Although the association constants are themselves assumed to vary with temperature there is no information regarding the variation of the ratio. If the ratio was independent of temperature, two simple predictions would follow. First, the initial rate of the relaxation process (calculated here as $k_{init} = A_r/\tau$) would be independent of the GDP concentration and second, the plot of the amplitude data as A_r vs. $(1+x)/x$ (see eq. 8) would be a straight line with intercept zero and slope equal to $F(C'_c - C_c)$. The predicted value for the slope may be obtained using the values of F and C_c derived by analysis of the full assembly process, and obtaining C'_c from the change in the critical concentration between 21.5 and 37°C estimated from the relaxation experiment performed in the absence of added GDP. On this assumption the slope is predicted to be 0.042.

The experiments show that neither of these predictions is correct. The initial rate is dependent

on the GTP/GDP ratio in a manner similar to that described by Engelborghs and Van Houtte [7] (see table 3); the plot of the amplitude data according to eq. 8 (fig. 5) does not have an intercept equal to zero and the slope is substantially less than 0.042. We therefore conclude that the ratio of the association constants is indeed dependent upon temperature.

Using F, C_c and C_c' derived above, by substitution in eq. 7, we find that $a' = 3.81$. Considering the range of possible values of a as discussed above, we believe that the upper and lower limits for the value of a' are 4.34 and 3.42. The value of a which we derive is in fact intermediate between the literature values of 2.8 and 4.34 (vide supra). Increasing the value of a tends to increase the slope and reduce the intercept of the plot according to eq. 8. Using $a = 6$ (cf ref. 2) gives a plot which closely matches the theoretical line with slope 0.038 (± 0.002) and intercept 0.0038 (± 0.0036). This alternative analysis would imply $a = a' = 6$. Re-analysis of the kinetic data using eq. 4 (with $a = 6$) gives a slope of 14.32 (± 1.22) $\times$ 10^{-3} s^{-1} and intercept -2.56 (± 0.79). Whereas the slope is acceptable within experimental error (see preceding discussion) the intercept is significantly different from zero $\pm$ experimental error. These discrepancies, together with the fact that the initial rate data for the relaxation experiment do show significant variation with GDP concentration (see table 3) (cf. ref. 7), lead us to reject the analysis that sets $a = a' = 6$.

One further question concerns the rate of nucleotide exchange. Engelborghs and Van Houtte [7] showed that whereas nucleotide exchange was fast at 35°C, it was slow enough to be rate-limiting at 25°C. An early study [20] reported that the half-life for GDP dissociation from the E-site was approx. 1 min, i.e., slow enough to interfere with the processes being measured here. However, later studies reported values for the rate of GDP release to be 0.14 s^{-1} [21] and 0.15 s^{-1} [22], corresponding to a half-life of about 5 s, which is rapid on the time scale of our experiments. We note that in the full assembly experiments the change in rate following addition of GDP is essentially instantaneous, consistent with the faster rate of exchange.

Based upon a reasonable presumption of a difference in the relative affinities of GTP and GDP over a temperature range of some 15 degrees, the model used here accounts for the observed variation in the initial rate of the relaxation experiment with GDP concentration. We therefore infer that the ratio of the association constants for nucleotide binding to tubulin is temperature dependent with values in the range 1.9–2.5 to 37°C and in the range 3.42–4.34 at 21.5°C. The relative affinity of GTP and GDP for tubulin therefore decreases by a factor of approx. 2 as the temperature is raised from 21.5 to 37°C. The additional relative free energy of GTP binding at 21.5°C would amount to $RT \ln 2 = 2 \times 294.5 \times \ln 2 = 400$ cal = 1.7 kJ. It appears entirely possible that such stabilisation could derive, say, from interaction of the terminal phosphate with charged groups on the protein and that this interaction would show some temperature dependence.

We therefore conclude that the effects of GDP on microtubules at steady state are treated adequately by this relatively simple mechanism. This includes rapid pre-equilibrium between tubulin-GTP and tubulin-GDP coupled to the elongation reaction, plus a reasonable variation with temperature in the ratio of the tubulin-nucleotide association constants. We therefore question the necessity to include more complex mechanisms in which GDP is assumed to modify the reactivity of microtubule ends.

Caplow and co-workers [23] have recently produced experimental evidence which requires a terminal tubulin-GDP subunit to accept elongation by tubulin-GDP, contrary to the original postulate of Carlier and Pantaloni [8]. The results presented here, together with theoretical arguments [13], support the view of Caplow et al. [23] since they suggest either that nucleotide exchange into the terminal positions of an elongating microtubule does not occur on the necessary time scale, or, if it does occur, that it is without marked effect on subsequent elongation reactions involving GTP-tubulin.

References

1 R.C. Weisenberg, W.J. Deery and P.J. Dickinson, Biochemistry 15 (1976) 4248.

2 M. Carlier and D. Pantaloni, Biochemistry 17 (1978) 1908.
3 R.K. MacNeal and D.L. Purich, J. Biol. Chem. 253 (1978) 4683.
4 T.L. Karr, A.E. Podrasky and D.L. Purich, Proc. Natl. Acad. Sci. U.S.A. 76 (1978) 5475.
5 R.V. Zackroff, R.C. Weisenberg and W.J. Deery, J. Mol. Biol. 139 (1980) 641.
6 L. Jameson and M. Caplow, J. Biol. Chem. 255 (1980) 2284.
7 Y. Engelborghs and A. Van Houtte, Biophys. Chem. 14 (1981) 195.
8 M. Carlier and D. Pantaloni, Biochemistry 21 (1982) 1215.
9 M. Carlier, Mol. Cell Biochem. 47 (1982) 97.
10 T.L. Hill and M. Carlier, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 7234.
11 Y.D. Chen and T.L. Hill, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 7520.
12 T.L. Hill and M. Carlier, Proc. Natl. Acad. Sci., U.S.A. 81 (1984) 5772.
13 P.M. Bayley and S.R. Martin, Biochem. Biophys. Res. Comm. 173 (1986) 351.
14 M.C. Shelanski, F. Gaskin and C.R. Cantor, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 765.
15 D.C. Clark, S.R. Martin and P.M. Bayley, Biochemistry 20 (1981) 1924.
16 P.M. Bayley, F.M.M. Butler, D.C. Clark, E.J. Manser and S.R. Martin, Biochem. J. 227 (1985) 439.
17 R.G. Burns and K. Islam, FEBS Lett. 173 (1984) 67.
18 B. Zeeberg and M. Caplow, Biochemistry 18, (1979) 3880.
19 J.L. Fishback and L.R. Yarbrough, J. Biol. Chem. 259 (1984) 1968.
20 B.J. Terry and D.L. Purich, J. Biol. Chem. 254 (1979) 9469.
21 B.P. Brylawski and M. Caplow, J. Biol. Chem. 258 (1984) 760.
22 Y. Engelborghs and J. Eccleston, FEBS Lett. 141 (1982) 78.
23 M. Caplow, J. Shanks and B.P. Brylawski, J. Biol. Chem. 260 (1985) 12675.