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Interactions of tubulin and microtubule-associated proteins

Conformation and stability of the oligomeric species from glycerol-cycled microtubule protein of bovine brain

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1. The conformation of bovine microtubule protein prepared by cycles of assembly and disassembly in the presence of glycerol has been studied by near-u.v. circular dichroism (c.d.) over a range of protein concentrations. The effects on the conformational properties of ionic strength and of a pH range from 6 to 7.5 have been correlated with the known oligomeric composition of microtubule protein preparations, as determined by the sedimentation behaviour of this preparation [Bayley, Charlwood, Clark & Martin (1982) *Eur. J. Biochem.* **121**, 579–585]. 2. The formation of 30S oligomeric ring species, either by decreasing ionic strength at pH 6.5 or by changing pH in the presence of 0.1 M-NaCl, correlates with a significant change in tubulin c.d. Formation of 18S oligomer by changing pH at ionic strength 0.2 produced no comparable effect. The c.d. of tubulin dimer itself is not affected by ionic strength and pH over the same range. 3. The results are interpreted as a small conformational adjustment between tubulin and specific microtubule-associated proteins on forming 30S oligomeric species, due to interaction with the high-molecular-weight-group proteins. The possible significance of this is discussed with respect to microtubule assembly *in vitro*. 4. By using this conformational parameter, together with equilibrium and kinetic light-scattering studies, the sensitivity of glycerol-cycled microtubule protein to dilution is shown to be strongly pH-dependent, the oligomers being much more stable at pH 6.4 than at pH 6.9. 5. Oligomeric complexes of tubulin with microtubule-associated proteins show marked stability under conditions similar to those for efficient microtubule assembly *in vitro*. Oligomeric material therefore must be incorporated directly during assembly *in vitro* from microtubule protein.

The isolation of the various proteins that comprise microtubules has been accomplished by several modifications of the two basic preparative techniques. The essentially classical biochemical fractionation (Weisenberg *et al.*, 1968; Weisenberg & Timasheff, 1970) yields tubulin dimer as a single homogeneous protein. By contrast, methods of preparation based on repeated cycles of temperature-mediated assembly and disassembly yield 'microtubule protein', a mixture of tubulin dimer and MAPs. These cycle preparations are generally modifications of the method described by Shelanski *et al.* (1973), which uses glycerol in all the assembly steps. Glycerol-free assembly–disassembly procedures have also been developed, for isolation of microtubule protein from porcine brain (Borisy *et al.*, 1975; Scheele & Borisy, 1979) and from bovine brain (Asnes & Wilson, 1979). The microtubule protein preparation contains free tubulin dimer and oligomeric complexes (composed of tubulin and MAPs) whose compositions depend on the preparative method and the conditions of concentration, pH and ionic composition. Tubulin dimer can be obtained from the cycled preparations with full retention of assembly properties by chromatography of microtubule protein under mild conditions on Sepharose 6B (Erickson, 1974; Clark *et*

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Abbreviations used: MAPs, microtubule-associated proteins; HMW MAPs, high-molecular-weight microtubule-associated proteins; standard buffer, 100 mM-Mes (4-morpholine-ethanesulphonic acid)/0.1 mM-EGTA/0.5 mM-$MgCl_2$; Pipes buffer, as for standard buffer, but with Mes replaced by 100 mM-Pipes (1,4-piperazinediethanesulphonic acid).

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al., 1981) or on phosphocellulose that has been presaturated with Mg^{2+} (Williams & Detrich, 1979).

The conformational properties of tubulin and microtubule protein have been studied by c.d. spectroscopy (Lee *et al.*, 1978, 1980; Clark *et al.*, 1980, 1981). The near-u.v. c.d. spectrum of microtubule protein from bovine brain is markedly different from that of dimer from the same source isolated by fractionation procedures (Clark *et al.*, 1980; Lee *et al.*, 1980). This difference is attributable to a difference in conformation of the tubulin dimer in the two preparations and not simply due to the presence of MAPs in the microtubule protein. Tubulin dimer isolated from microtubule protein by chromatography has a spectrum similar to that of the microtubule protein itself (Clark *et al.*, 1981). However, this dimer preparation does show small but reproducible differences from the spectrum of the microtubule protein. We investigate in the present paper whether the conformation of the tubulin dimer is significantly modulated in the microtubule protein preparation due to interaction with the MAPs. Since these proteins have a marked influence on the assembly characteristics of microtubule protein *in vitro*, this enables an assessment to be made of the role of protein conformation in microtubule assembly.

The cold dissociation of microtubules formed from the glycerol-free microtubule protein from porcine brain is known to produce a mixture of 6S tubulin dimer and two oligomeric species with $s^0_{20,w}$ values of 18S and 30S (Scheele & Borisy, 1976; Marcum & Borisy, 1978). The relative proportions of these species are determined by pH, ionic strength and protein concentration. The sedimentation properties of glycerol-cycled microtubule protein from bovine brain were recently shown to be similar to, though not identical with, those of glycerol-free porcine microtubule protein (Bayley *et al.*, 1982). In the present paper, the c.d. spectrum of the bovine brain microtubule protein prepared in the presence of glycerol is examined under conditions of pH, salt and protein concentrations known to influence the distribution of oligomeric forms, to correlate conformational properties with the oligomeric composition. Control experiments have been performed with tubulin dimer from chromatography on Mg^{2+}-presaturated phosphocellulose, in order to examine directly the small differences between the dimer and the microtubule protein preparation. Also the stability has been assessed of the oligomeric species under conditions relating to the assembly of microtubules from microtubule protein preparations *in vitro*.

Materials and methods

Bovine brains were obtained from British Beef, Watford, Herts., U.K. GTP (type IIs) and ATP were purchased from Sigma Chemical Co. Phosphocellulose (Whatman P11) was swollen in water and washed with 50% ethanol, 0.5M-NaOH, water, 0.5M-HCl and finally water as described by Weingarten *et al.* (1975). All other chemicals were AnalaR grade and were used without further purification.

Microtubule protein was prepared by cyclization in the presence of glycerol as described previously (Clark *et al.*, 1981). Tubulin dimer was prepared by chromatography of microtubule protein by the method of Williams & Detrich (1979). Solutions for study by c.d. spectroscopy were prepared in standard buffer adjusted to the appropriate pH (with NaOH or HCl) and ionic strength (with NaCl).

C.d. spectra were recorded on a Jasco J41C spectropolarimeter equipped with a model J-DPY Data Processor. Fused-silica cells of pathlength 2, 5, 10 and 40mm were used as appropriate to record spectra from 340 to 250nm, at a sensitivity of 0.5 m-degree/cm, with an instrumental time constant of 16s. The spectra shown here are averages of at least two scans and are presented as molar c.d., $\Delta\varepsilon$, based on a mean amino acid-residue mol.wt. of 110. The mean residue ellipticity may be obtained from:

$$[\theta]_{m.r.w.} = 3300\,\Delta\varepsilon$$

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by a modification of the method of Laemmli (1970), with quantitative analysis of stained gels performed as previously described (Clark *et al.*, 1981). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Equilibrium-light scattering measurements were performed at 90° with light of $\lambda = 577$nm in an apparatus incorporating a 10W argon ion laser, as used for quasi-elastic light scattering (Sattelle *et al.*, 1982; G. A. Palmer, D. C. Clark, D. B. Sattelle & P. M. Bayley, unpublished work). Calibrated filters were used to vary the intensity of the incident light and to enable a wide range of protein concentrations to be covered. Solutions of microtubule protein were clarified by centrifugation for 45min at 100000*g* and carefully diluted under dust-free conditions with buffer filtered through 0.1μm millipore filters. Solutions were equilibrated at 5°C before measurement.

Kinetic light-scattering measurements were performed at 90° with light of $\lambda = 400$nm (2nm spectral bandwidth) in a Perkin–Elmer MPF-4 spectrofluorimeter. A small volume of concentrated protein solution was added to buffer in a 1cm cuvette to give a 10-fold dilution at a known pH, and the scattered light intensity was followed as a function of time for a period of approx. 10min. The analogue output of the fluorimeter was recorded on the chart and was also 'digitized' directly into a

PDP11/23 computer for subsequent analysis by the non-linear least-squares procedures as described by Bevington (1969).

Stopped-flow measurements were made by using 180° observation of transmitted light to monitor turbidity changes after rapid mixing of microtubule protein and salt solutions at 20°C. The system was as described by Bayley (1981), using light of $\lambda = 436$ nm from a 100W high-pressure mercury arc. The data were collected in a transient recorder (DL905) and transferred to the main DEC-20 computer for on-line analysis as above.

Results

C.d. spectra

The near-u.v. c.d. spectrum of the microtubule protein preparation at 1.28mg/ml has been recorded as a function of pH in the range 6.38–7.29 (Fig. 1). The spectrum at pH6.38 is similar to, though slightly more intense than, that previously reported for microtubule protein (Clark *et al.*, 1981). Increasing the pH leads to a progressive loss of intensity at 280nm ($\Delta\varepsilon_{280}$ changes from $-0.015\,M^{-1}\cdot cm^{-1}$ at pH6.38 to $-0.0105\,M^{-1}\cdot cm^{-1}$ at pH7.29). Spectra recorded at higher pH values (up to pH7.7) are very similar to that shown for pH7.29. Thus, the majority of the total change occurs between pH6.38 and 7.05.

Absorption and near-u.v. c.d. spectra have also been recorded for the microtubule protein preparation (at 2.65mg/ml) as a function of pH (6.4, 6.95 and 7.5) in standard buffer and in this buffer containing 0.1M-NaCl. The absorption spectrum at pH6.4 in the presence of NaCl shows substantially less light scattering than that observed in the absence of NaCl at the same pH. Further, in the presence of NaCl, the extent of light scattering is effectively unaltered by changes in pH (i.e. A_{330} is constant). By contrast, in the absence of NaCl, the extent of light scattering is substantially reduced as the pH is increased. Thus the ratio $A_{330}(-NaCl)/A_{330}(+NaCl)$ decreases progressively from 1.66 at pH6.4 to 1.09 at pH7.5. Fig. 2 shows the near-u.v. c.d. spectra recorded in the presence of 0.1M-NaCl and the behaviour parallels that observed with the absorption spectra. In the presence of NaCl the spectrum is effectively unaltered by increases in pH; the value of $\Delta\varepsilon_{280}$ is $-0.0115 \pm 0.0005\,M^{-1}\cdot cm^{-1}$ for each spectrum. The control experiments at 2.65mg/ml in the absence of added salt show effects identical with those of Fig. 1, but of lower overall amplitude.

The effect of a change in ionic strength alone has been investigated at several different protein concentrations. Fig. 3(*a*) compares the spectrum of microtubule protein at 8.8mg/ml (pH6.4) in the absence of added salt and in the presence of 0.1M- and 0.3M-NaCl. The results clearly parallel the

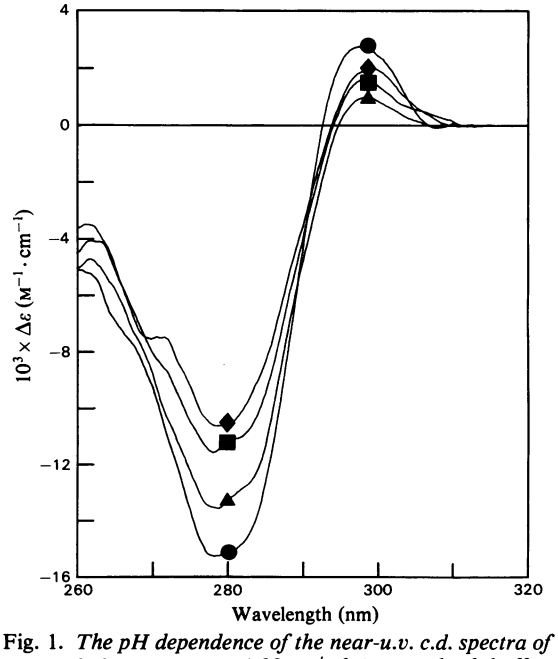

Fig. 1. *The pH dependence of the near-u.v. c.d. spectra of microtubule protein at 1.28mg/ml in standard buffer, pH6.4 (●), pH6.8 (▲), pH7.05 (■) and pH7.3 (♦)*

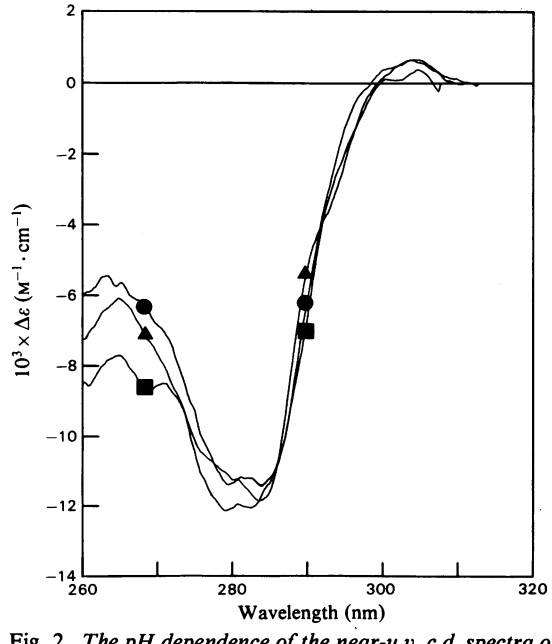

Fig. 2. *The pH dependence of the near-u.v. c.d. spectra of microtubule protein at 2.65mg/ml in standard buffer plus 0.1M-NaCl, pH6.35 (●), pH6.95 (▲) and pH7.5 (■)*

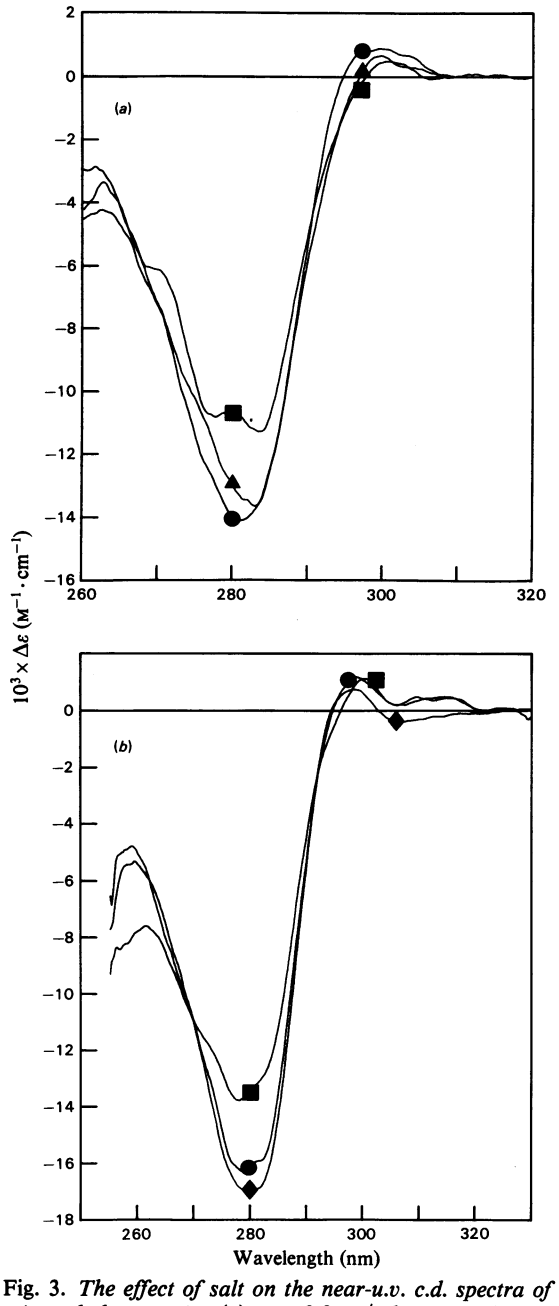

Fig. 3. *The effect of salt on the near-u.v. c.d. spectra of microtubule protein (a) at 8.8 mg/ml at pH 6.4 in standard buffer (●), in buffer plus 0.1 M-NaCl (▲) and in buffer plus 0.3 M-NaCl (■) and (b) at pH 6.5 in standard buffer at 0.2 mg/ml (●) and 4.0 mg/ml (♦) and in standard buffer at 0.2 mg/ml in the presence of 0.2 M-NaCl (■)*

effects of increasing pH noted above, $\Delta\varepsilon_{280}$ decreasing from $-0.015\,M^{-1} \cdot cm^{-1}$ in the absence of salt to $-0.011\,M^{-1} \cdot cm^{-1}$ in the presence of 0.3 M-NaCl. The value of $\Delta\varepsilon_{280}$ for the microtubule protein appears to be effectively independent of protein concentration over the range 1–9 mg/ml. However, because of the substantial variability (±15%) we find in the value of $\Delta\varepsilon_{280}$ from different preparations, the concentration dependence of the c.d. spectrum was carefully examined for a single protein sample. Fig. 3(*b*) shows that microtubule protein in standard buffer at 4.0 mg/ml has a c.d. spectrum identical with that at 0.2 mg/ml, confirming that the c.d. spectrum is independent of concentration over a very wide range. Also, even at 0.2 mg/ml, the effect of 0.2 M-NaCl is to reduce the c.d. intensity, as shown for the higher protein concentration in Fig. 3(*a*).

As a control, the effects of pH and ionic strength were examined for the tubulin dimer preparation, which is free of MAPs. The spectrum of the dimer is unchanged by increasing the pH to 6.9 or 7.4 and by the addition of 0.1 M-NaCl at pH 6.4 (Fig. 4*a*). Thus the effects of pH and ionic composition on the c.d. spectrum of the microtubule protein cannot be accounted for in terms of the effect of solution conditions on tubulin dimer itself, but must reflect the changes in molecular interactions between the components of the microtubule protein complex.

The c.d. spectrum of microtubule protein shows some variability from preparation to preparation, particulary at 260 nm (Clark *et al.*, 1981). Possible explanations are that this difference could arise from differences in MAP content, or from differences in total nucleotide content. The latter possibility was investigated by recycling two identical samples of protein, in the presence of 4 M-glycerol including 0.8 mM- or 0.08 mM-GTP. Comparison of the absorption spectra of the products revealed that substantially more nucleotide (approx. 40–50 μM) was carried through in the pellet after the assembly at the high nucleotide concentration. The near-u.v. c.d. spectra for these two samples are shown in Fig. 4(*b*). There is a significant difference between the two samples concentrated principally in the 260 nm region, consistent with a contribution from the nucleotide. Given the value of $\Delta\varepsilon_{260} = -0.6\,M^{-1} \cdot cm^{-1}$ for free GTP, the presence of various amounts of free nucleotide could account for the magnitude of the differences at 260 nm. However, it must be stressed that the nucleotide makes a barely detectable contribution to the c.d. at 280 nm, and different nucleotide contents cannot be responsible for the small differences between tubulin dimer and microtubule protein in this region. Indeed the spectra shown in Figs. 1–3 confirm that consistent changes are induced at 280 nm by variation of pH or ionic composition for microtubule protein at constant nucleotide composition.

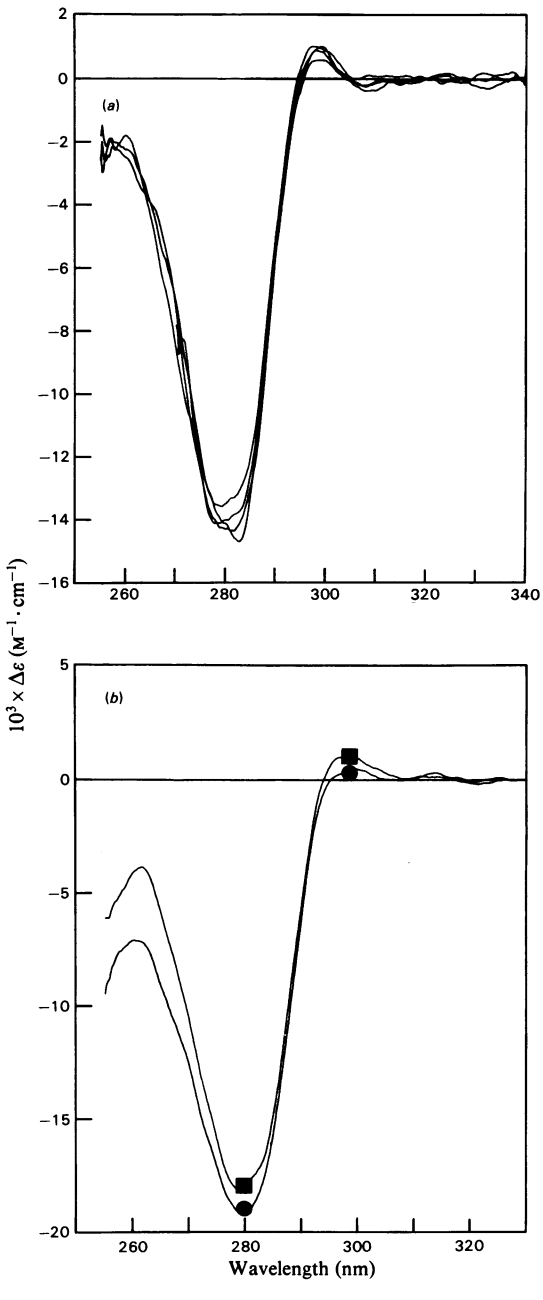

Fig. 4. *Near-u.v. c.d. spectra of tubulin dimer and microtubule protein: control experiments*

(*a*) Shows near-u.v. c.d. spectra of tubulin dimer (1.0mg/ml) at pH6.5, 6.9 and 7.35 in standard buffer and at pH6.5 in standard buffer plus 0.2M-NaCl. All curves superimpose within the experimental noise level, and are not distinguished. (*b*) Shows near-u.v. c.d. spectra of microtubule protein (1.8mg/ml) re-assembled in 4M-glycerol in the presence of 0.8mM-GTP (●) and in the presence of 0.08mM-GTP (■).

Light scattering

The intensity of light scattered by a solution of microtubule protein (under non-assembly conditions) provides a qualitative index of the proportion of the protein present as oligomeric species. The stability of the microtubule protein to dilution was measured by using laser light scattering (Fig. 5). Serial dilutions were made of the protein at pH6.5 by using standard buffer at 5°C, with light-scattering intensity monitored as described in the Materials and methods section. By using the laser light-scattering system, quantitative measurements could be made over a wide concentration range. The results indicate that a significant decrease in specific intensity is indeed observed, with a midpoint of transition at approx. 0.3mg/ml.

Studies of the kinetics of dissociation after dilution were performed in the fluorimeter (see the Materials and methods section). In addition to the standard buffer (pH6.5), a second buffer was also used in which Mes was replaced by 100mM-Pipes (pH6.5 or 6.94). This latter buffer is similar to that used in the assembly and preparation of porcine microtubule protein at pH6.94 in the absence of glycerol (Olmsted & Borisy, 1975; Borisy *et al.*, 1975). Typical traces of the time course of the scattered-

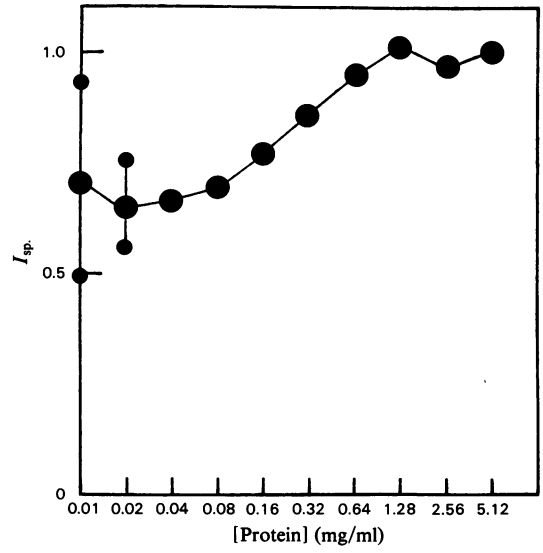

Fig. 5. *Normalized light-scattering intensity ($I_{sp.}$) of microtubule protein as a function of dilution*

$I_{sp.}$ is the observed light-scattering intensity divided by protein concentration. Standard buffer, pH6.5, was used, at 5°C with $\theta = 90°$. Data-collection time varied from 20s for the most concentrated sample to 100s for the most dilute, with a fixed sample time of 10μs. Laser power varied from 20mW for the most concentrated sample to 600mW for the most dilute.

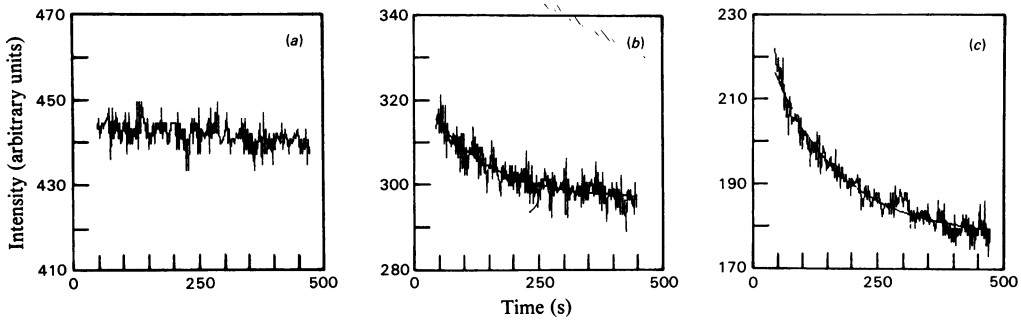

Fig. 6. *The time-dependence of intensity of scattered light (arbitrary units) after a 10-fold dilution of microtubule protein to a final concentration of 1 mg/ml and at 16°C*

(*a*) Protein in standard buffer, pH 6.5, diluted into the same buffer, pH 6.5. (*b*) As (*a*) pH 6.75. (*c*) Protein in Pipes buffer, pH 6.94, diluted into the same buffer. The smooth curves are single exponential fits to the raw original data (see the Materials and methods section) and the parameters are given in Table 1 (*a–c*).

Table 1. *Time-dependent changes in light scattering after dilution of microtubule protein at pH 6.5–6.9 at 16°C*

The protein stock solution (10 mg/ml), in the buffer indicated in parentheses, was diluted 10-fold on addition to the diluting solution. A single exponential process was analysed (see the Materials and methods section) as:

$$I(t) - I(\infty) = \Delta I \cdot e^{-kt}$$

(i.e. positive ΔI refers to a decrease in intensity) where units of I are arbitrary. Where no value of the rate is given, k was poorly determined owing to the smallness of the corresponding ΔI.

Stock-protein pH	Diluting-solution pH	Rate, k (s^{-1})	Plateau, I_∞	Amplitude, ΔI	$\Delta I/I_\infty$ (%)
(*a*) 6.5 (standard)	6.5 (standard)	—	440	10	2
(*b*) 6.75 (standard)	6.75 (standard)	0.008	297	27	11
(*c*) 6.94 (Pipes)	6.94 (Pipes)	0.008	177	51	30
(*d*) 6.5 (Pipes)	6.5 (Pipes)	—	183	2	1

light intensity are shown in Fig. 6. In Table 1 are presented the results from dilution experiments using a series of buffers and pH values. The analysis as a single exponential decay accounts satisfactorily for the observed process within the limits of the experimental data.

Table 1 (*a–c*) shows that dilution at constant pH causes a time-dependent decrease in scattered-light intensity whose amplitude increases markedly from pH 6.5 to 6.94. The equilibrium plateau value after dilution decreases markedly over the same pH range. Thus at the higher pH, the equilibrium oligomeric content is substantially less (at 1.0 mg/ml) and is much more sensitive to dilution. The experiments also show that, although the oligomeric composition is dependent on pH and protein concentration, it is also dependent on the buffer ion that is present. Thus dilution of the protein at pH 6.5 in Pipes buffer (Table 1*d*) causes only a small transient amplitude, but the equilibrium value is much less than in Mes at the same pH. These observations are all made on the glycerol-cycled bovine microtubule protein; essentially similar results are obtained with microtubule protein prepared from bovine or porcine brain in the absence of glycerol (P. M. Bayley, D. C. Clark & S. R. Martin, unpublished work). Dilution-induced dissociation of microtubule protein is clearly a slow process with rates occurring in the range 0.008–0.003 s^{-1} (i.e. a half life of 125–300 s) and with amplitudes that are strongly pH-dependent. The rate of dissociation after a pH change at constant protein concentration has not been examined explicitly in these experiments. If a dilution is made from a concentrated solution into buffer at a different pH, there is some indication of a fast process (preceding the slower process analysed in Table 1) that depends on the pH change.

Since assembly studies *in vitro* are generally performed at an elevated temperature, a parallel series of experiments was conducted at 30°C where addition of GTP (1 mM) caused rapid assembly of the microtubule protein at 1 mg/ml. Diluting solutions contained GDP (0.2 mM) to prevent assembly. In all cases the amplitudes observed were less

Table 2. *The effect of salt on the rate of dissociation of microtubule protein observed after stopped-flow mixing*
Results are means ± S.D. for the numbers of determinations shown in parentheses.

Protein sample	[Protein] (mg/ml)	$k_{obs.}$ (s^{-1}) 0.2 M-NaCl	0.4 M-NaCl	0.8 M-NaCl
Microtubule protein	0.55	17.0 ± 3.2 (9)	18.9 ± 5.0 (5)	15.6 ± 2.5 (6)
+ GTP (0.1 mM)	0.55	14.0 ± 2.9 (10)	—	17.2 ± 5.7 (4)
+ GDP (0.1 mM)	0.55	17.2 ± 5.9 (13)	16.7 ± 4.2 (5)	14.7 ± 5.9 (4)

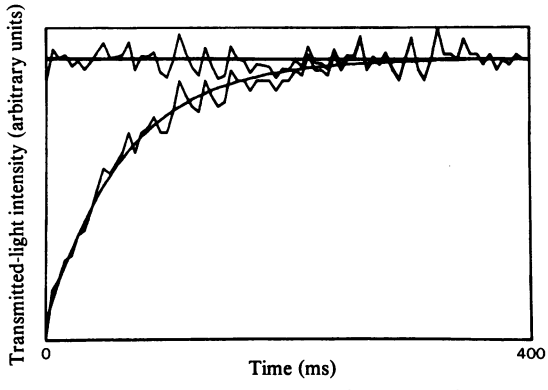

Fig. 7. *A light-scattering stopped-flow trace for microtubule protein (0.55 mg/ml) in 0.4 M-NaCl*
Total time = 400 ms; $k_{obs.} = 16.4\,s^{-1}$. The Figure shows the experimental and fitted curve and the residual difference.

than those recorded in Table 1 (*a–c*), indicating even less tendency to dissociate under solution conditions closely similar to those used in assembly assays.

Since salt is known to promote dissociation of microtubule protein, the effect of increasing salt concentration on the kinetics of dissociation was studied using stopped-flow mixing with light-scattering detection. A typical transient is shown in Fig. 7, representing the decrease in scattered-light intensity (increase in transmission) observed after the stopped-flow mixing of equal volumes of protein and salt solutions. The final protein concentration was 0.55 mg/ml and final salt concentration was 0.2, 0.4 or 0.8 M. No systematic dependence on salt concentration was detected (Table 2) and the rate constant ($18.5\,s^{-1}$) was not significantly affected by the presence of 10^{-4} M-GTP or -GDP ($14.9\,s^{-1}$ and $16.6\,s^{-1}$ respectively). It may be noted that these rates are much faster than dissociation rates due to dilution alone. In all cases the amplitude represented 10–20% of the equilibrium turbidity at $t = \infty$. Slower processes were not observed.

Discussion

The conformational properties of tubulin dimer are clearly of importance as a potential factor involved in the assembly of microtubules. The recognition of the oligomeric complexes formed between tubulin and MAPs, and the possible role of these oligomers in the assembly process, requires tubulin conformation to be assessed in both the presence and absence of MAPs. Further, the oligomers provide a simple model system for studying the interactions between tubulin and MAPs, interactions that are of considerable importance in forming and stabilizing assembled microtubules. It is known that these oligomeric species are not obligatory intermediates in the unseeded or seeded assembly of tubulin *in vitro* (Frigon & Timasheff, 1975; Bryan, 1976; Lee & Timasheff, 1977; Johnson & Borisy, 1977), which proceeds by the condensation–polymerization of tubulin dimer (Oosawa & Asakura, 1975). By contrast, the role of oligomers of tubulin dimer and MAPs in assembly of microtubules from preparations of microtubule protein remains controversial.

The present results show significant variation of the near-u.v. c.d. properties of microtubules protein as a result of changing solution conditions over a range known to affect the distribution of molecular species. As has previously been shown by Marcum & Borisy (1978) for porcine brain microtubule protein (prepared in the absence of glycerol by cycles of assembly–disassembly), glycerol-cycled bovine brain microtubule protein also contains three main components: '6S dimer' (the tubulin $\alpha\beta$ heterodimer, M_r 110000) and two oligomeric species containing tubulin and MAPs ('18S oligomer' and '30S ring' species) (Bayley *et al.*, 1982). The relative proportions of all three species are markedly sensitive to protein concentration, ionic composition of the buffer and pH. Thus for concentrations of 2 mg/ml and above, in the standard buffer, raising the pH causes a marked change in the oligomeric composition. At pH 7 only the 30S ring species is found, whereas at pH 7 and above, 18S oligomer predominates. In this buffer plus 0.1 M-NaCl, the 30S ring species is not found but, above pH 6.5, the

18S oligomer is effectively the exclusive oligomeric species at almost all protein concentrations. However, unlike 30S ring, the 18S oligomer is clearly unstable at protein concentrations less than 2mg/ml. Thus by manipulating the solution conditions, the microtubule protein can be studied under conditions where either 30S or 18S species predominates, together with a substantial proportion of tubulin present as the 6S dimer.

These considerations permit a clear interpretation of the near-u.v. c.d. results presented here. Under the different buffer conditions the species present change from (6S+30S) to 6S over the range pH6.4–7.3 with protein concentration 1.28mg/ml (Fig. 1), (6S+30S) to (6S+18S) over the range pH6.35–7.5 at 2.65mg/ml and 6S to (6S+18S) over the range pH6.35–7.5 in the presence of 0.1M-NaCl at a protein concentration of 2.65mg/ml (Fig. 2). In all cases the disappearance of the 30S species is directly associated with the decrease in $\Delta\varepsilon_{280}$, whereas formation of 18S oligomer from 6S dimer shows no c.d. changes. Likewise, the increasing ionic strength at constant pH, the disappearance of 30S species (with increasing [NaCl]) shows a decrease in $\Delta\varepsilon_{280}$ (Fig. 3; 8.8mg/ml). Under a range of pH and salt conditions tubulin dimer prepared by phosphocellulose chromatography shows no variation in its c.d. spectrum and the values observed are close to those for microtubule protein under conditions of pH and ionic composition leading to the full dissociation of 30S oligomeric species. [Progressive changes in the c.d. of tubulin dimer accompanying pH increases above 7.5 have been fully documented by Lee *et al.* (1978).] Decreased light scattering at $\lambda > 300$nm under corresponding conditions as ionic strength or pH is increased are also consistent with the disappearance of the 30S oligomeric ring species.

The role of conformation in the processes of breakdown and interconversion of the oligomeric species of the microtubule protein preparation studied here is clearly important in considering the ability of the MAPs to promote efficient assembly *in vitro*. In interpreting the change of the $\Delta\varepsilon_{280}$ observed here, a number of models are possible. (1) The tubulin c.d. remains constant; MAPs undergo a conformational change. Since the approximate composition of this bovine microtubule protein prepared in the presence of glycerol is 85% tubulin and 15% MAPs (Bayley *et al.*, 1982), this would require MAPs to have a c.d. some 3-fold more intense than tubulin itself. Preliminary measurements on crude total MAP fraction indicated a low aromatic amino acid content, together with a very weak c.d., and this model appears improbable. (2) Tubulin c.d. is enhanced in the 30S oligomer; the c.d. of MAPs remains very weak. This enhancement could be due either to the direct effect of the MAPs on appropriate aromatic residues, in the manner of a small ligand interacting directly with aromatic side chains close to a rigid binding site (Goux & Hooker, 1980), or the tubulin conformation could be changed on interaction with the macromolecular ligand (MAPs), possibly by reorganization of protein domains. This latter inference provides a reasonable and simple working model for this process. It is also consistent with observations that tubulin itself has a readily perturbed conformation, as indicated by its sensitivity to non-aqueous solvents (P. M. Bayley, D. C. Clark & S. R. Martin, unpublished work). From reconstitution experiments, it appears most likely that it is the HMW MAPs that are involved in forming the 30S species, and are therefore implicated in these interactions (Vallee & Borisy, 1978).

In the work of Borisy and his co-workers with glycerol-free porcine microtubule protein, a dynamic equilibrium was inferred between various species at pH6.94, since, in analytical ultracentrifugation at 5°C, the 30S species was found to dissociate to 6S material at a total protein concentration 1mg/ml (Scheele & Borisy, 1979). Thus, in addition to possible pressure effects on the equilibrium, this 30S ring species appears intrinsically unstable at lower total protein concentrations. By contrast, the observed constancy of the c.d. spectrum of the glycerol-cycled bovine microtubule protein over a 20-fold range of protein concentration, and the ability to demonstrate the full 'salt effect' at 0.2mg/ml (Fig. 3*b*) indicates that the 30S species shows little tendency to dissociation by dilution in standard buffer at pH6.5. This has been confirmed directly in the equilibrium experiments by using laser light scattering (Fig. 5). The measurements of the time-dependent changes on dilution extend these observations. At higher pH, the glycerol-cycled bovine microtubule protein shows dissociation on dilution, i.e. it behaves more like the glycerol-free porcine microtubule protein of Marcum & Borisy (1978). These measurements also show that dissociation is critically dependent on pH over a narrow range, in full agreement with the conclusions from the c.d. measurements, with the oligomeric composition readily perturbed at pH6.94 but effectively independent of concentration at pH6.5 (Fig. 6). Since similar results have been obtained for glycerol-free microtubule protein from bovine and porcine brain (P. M. Bayley, D. C. Clark & S. R. Martin, unpublished work), the sensitivity to dilution appears unrelated to the inclusion of glycerol in the preparative procedure. Thus apparent differences in the stability to dilution of microtubule protein prepared by various methods are correlated with the different solution conditions employed. This may explain differences in the effects of dilution plus changes in temperature on the equilibria of the oligomeric ring

structures as observed by Engelborghs *et al.* (1980) and Karr & Purich (1980). Their models, which ignore the high pH sensitivity of the stability of oligomeric species, must bear only indirectly on general mechanisms of self-assembly.

It is well recognized that interactions between tubulin and MAPs are strongly perturbed by increased ionic strength. Quasi-elastic light scattering showed a decrease in scattered-light intensity and an increase in mean diffusion coefficient, though both parameters showed changes considerably less than expected for full molecular dissociation (G. R. Palmer, D. C. Clark, D. B. Sattelle & P. M. Bayley, unpublished work). Analytical ultracentrifugation showed that the main species present in 0.1 M-NaCl is in fact the 18S oligomer (Bayley *et al.*, 1982), as previously observed by Marcum & Borisy (1978) for glycerol-free porcine microtubule protein. The speed of the salt-induced process suggests that changes in ionic composition represent a highly efficient means of changing the nature of interactions between tubulin and MAPs and hence in affecting a change in oligomeric composition possibly in favour of the 18S species, given appropriate ionic conditions.

It is interesting that, in addition to pH, the nature of the buffer ion is also an important determinant of stability of the oligomer. At pH 6.5, standard buffer stabilizes the oligomer to a greater extent than Pipes buffer of the same concentration. Given the differences in p*K* for Mes (6.1) and Pipes (6.8), the former would be predominantly anionic and the latter cationic, though both, of course, are zwitterionic buffers. This suggests that for the oligomeric species *in vitro*, and, by extrapolation, possibly for assembled microtubules *in vivo*, the stability and hence integrity of the structure might be modulated by relatively small changes in local ionic conditions. It has previously been noted that polycations can substitute for MAPs, apparently in a non-specific way, in lowering the critical concentration for microtubule assembly *in vitro*. It is interesting to speculate that they may serve in a more specific manner by affecting the stability of interactions between tubulin dimer and MAPs, and hence contribute to the regulation of the microtubule system *in vivo*.

The role of ring-type oligomers in the assembly of microtubule protein *in vitro* is still controversial. The 30S ring species is clearly stable at pH 6.5 and at temperatures of 20–30°C, so that there would be little dissociation to tubulin plus MAPs during the lag-phase of assembly. Thus the question may be posed whether rings are involved directly as such in assembly or involved indirectly in producing an oligomeric intermediate effective either in the nucleation/initiation phase, or in the growth/elongation phase (cf. Mandelkow *et al.*, 1981). It was shown (Clark *et al.*, 1981) that, compared with the fractionated tubulin dimer (Weisenberg & Timasheff, 1970), tubulin dimer prepared by phosphocellulose chromatography (in the presence of Mg^{2+}) is highly efficient in an assembly assay seeded by 30S ring material. The c.d. results presented here show that there is little conformational difference between free tubulin dimer prepared in this way and that present in the oligomeric 18S species. Little is known of the structure of this species, except that it appears not to be a 'ring' form. It may well be simply an open form of tubulin oligomer, stabilized by interactions with specific MAPs. Given its conformational identity with the tubulin dimer from magnesium phosphocellulose chromatography, such an intermediate species, derived from 30S oligomer, could be highly effective in assembly of microtubule protein by presenting an oligomeric and hence multivalent complex of tubulin in a conformation suitable for efficient incorporation in either the nucleation or elongation stages of the assembly process *in vitro*. We conclude that the direct incorporation of oligomeric material must contribute significantly to the mechanism of microtubule assembly *in vitro* from microtubule protein, in addition to assembly via the pathway of condensation–polymerization of tubulin dimer.

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