Differences in polypeptide composition and enzyme activity between cold-stable and cold-labile microtubules and study of microtubule alkaline phosphatase activity

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Cold-stable and cold-labile microtubules were prepared by two cycles of assembly and disassembly and two periods of exposure to cold. The cold-labile preparations were shown to contain a higher proportion of a high molecular mass microtubule-associated protein (MAP 2) than cold-stable preparations. The cold-stable preparations showed a much higher alkaline phosphatase activity. Stimulation of microtubule assembly by zinc led to increases in both cold stability and alkaline phosphatase activity.

Microtubule *Cold stability* *Microtubule-associated protein* *SDS-gel electrophoresis* *Alkaline phosphatase* *Zinc*

1. INTRODUCTION

Microtubules are thought to be important in several important cell functions, such as mitosis and intracellular transport [1]. Isolated microtubules have been shown to consist primarily of tubulin together with small quantities of microtubule-associated proteins (MAPs) of which the high molecular mass MAP 1 and MAP 2 and the tau family of proteins are the best characterized [2–4]. Although microtubule formation from tubulin and MAPs can be studied in vitro, the factors regulating microtubule assembly and stability are not understood. It has been found that brain extracts contain a proportion of microtubules which are stable to cold [5–7]. Study of cold-labile and cold-stable microtubules is one approach to investigate the factors involved in regulating microtubule stability and it is therefore important to characterize these two types of microtubules in terms of their polypeptide composition and enzyme activities.

Using a variety of isolation procedures 3 groups of workers have studied cold-stable microtubules; two have reported no difference in polypeptide pattern between cold-stable and cold-labile microtubules [5,6], whereas the third group has reported the presence of minor components unique to the cold-stable microtubules [7]. In addition the latter group has found cold stability to be correlated with the presence of a phosphorylated 64 kDa protein [7] and more recently to be regulated by both calmodulin-dependent and calmodulin-independent phosphorylation reactions of other components [8,9]. This paper describes the isolation of cold-stable and cold-labile microtubules from rat brain by two cycles of assembly and disassembly and the differences between these preparations in their microtubule-associated protein components and their alkaline phosphatase activity. In addition, since stimulation of microtubule assembly in rat brain extracts by zinc has been shown to cause an increase in microtubule cold stability, its effects on alkaline phosphatase activity have been investigated.

2. MATERIALS AND METHODS

2.1. Materials

Piperazineethanesulphonic acid (Pipes), *p*-nitrophenol phosphate and GTP were purchased from Sigma. Standard laboratory chemicals were Analar

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grade from BDH. Male rats of the Rowett Hooded Lister strain, weighing approx. 200 g, were used.

2.2. Preparation of microtubules

Cold-stable and cold-labile microtubules were prepared from rat brain by 2 cycles of assembly/disassembly and exposure to cold, as outlined in fig.1. Brains were homogenized in ice-cold 100 mM Pipes buffer (pH 6.9), 1 mM EGTA (buffer H). The cold-labile material (supernatant) was collected from the microtubules assembled from the first cycle cold-labile material. The cold-stable material (pellet) was collected from the microtubules assembled from resuspended first-cycle cold-stable material; it was resuspended in buffer H, homogenized and the microtubules were sheared by passage through a needle. Samples were stored at −20°C before analysis. Samples were dialysed for 3 h against 100 vols of 0.2 M glycine–NaOH buffer (pH 10.4) prior to measurement of alkaline phosphatase activity.

2.3. Zinc-stimulation of assembly

Supernatant fluids from rat brain were prepared by centrifuging brain homogenates at 100000 × *g* for 30 min in the cold [10,11]. Assembly was allowed to occur by warming the fluid at 37°C for 50 min in the absence of added guanine nucleotide [5,11], either without added zinc or in the presence of 500 μM zinc chloride. After the incubation period, microtubules were collected by centrifugation at 100000 × *g* for 30 min at 20–24°C and resuspended in 100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA (pH 6.9). In some experiments aliquots were dialysed against 100 vols of 100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA (pH 6.9) prior to measurement of alkaline phosphatase activity. In a second series of experiments microtubules were assembled in the absence of zinc, resuspended in 100 mM Pipes buffer (pH 7.0), incubated with either no added cations, Mg^{2+} (5 mM) or Zn^{2+} (500 μM) for 2 min and then frozen at −20°C, either directly or after analysis for 3 h against 100 vols of 100 mM Pipes, 1 mM EGTA (pH 6.9).

2.4. Other methods

Alkaline phosphatase activity was measured in 0.2 M glycine–NaOH buffer (pH 10.4) using 5 mM *p*-nitrophenol phosphate as substrate [12]. Incubations, containing 0.1–0.5 mg protein, were at 37°C for 1 h after a 5 min pre-incubation without substrate. Blanks, both reaction blanks without sample and sample blanks with sample but without *p*-nitrophenol phosphate, were carried out in parallel and the results corrected accordingly. The reaction was stopped by addition of 2 ml of 0.25 N NaOH and the absorbance measured at 420 nm. The molar extinction coefficient of *p*-nitrophenol was taken as 14330 at 420 nm [12]. SDS–polyacrylamide gel electrophoresis was carried out using discontinuous slab gels and Tris–glycine buffer [13]. Samples were heated for 4 min at 100°C in the presence of 1% SDS and 10% mercaptoethanol. Gels were stained with Coomassie brilliant blue, destained in methanol–acetic acid and scanned in a Zeinah densitometer. Relative amounts of the major components were calculated from measurements of peak areas. Protein was measured as in [14].

3. RESULTS

Cold-stable and cold-labile microtubules were prepared by two cycles of assembly and disassembly as shown in fig.1. This scheme essentially uses the classical assembly/disassembly method of microtubule preparation [15] with the addition of two periods of exposure to cold and subsequent separation of cold-stable and cold-labile microtubules by centrifugation. Electron microscopy showed microtubules to be present in the cold-stable material (not shown). SDS-polyacrylamide gel electrophoresis (fig.2) showed both cold-stable and cold-labile preparations to exhibit a polypeptide composition similar to that of microtubule protein [2–4]; the major component was tubulin together with a doublet of high molecular mass proteins (>300 kDa), MAP 1 and MAP 2, and other minor components in the 60–80 kDa range. However, differences in the composition of the minor components were observed. Firstly, more of one of the high molecular mass MAP bands was present in the cold-labile preparations (fig.2a); this band co-migrated with MAP 2. Densitometry confirmed that this component represented a greater proportion of the protein in the cold-labile microtubules (fig.2b); measurement of peak areas from scans of 4 pairs of preparations showed that the MAP 2 composed approx. 13% ± 3% (SE) of the total protein in the cold-stable parations, whilst the content of the presumptive

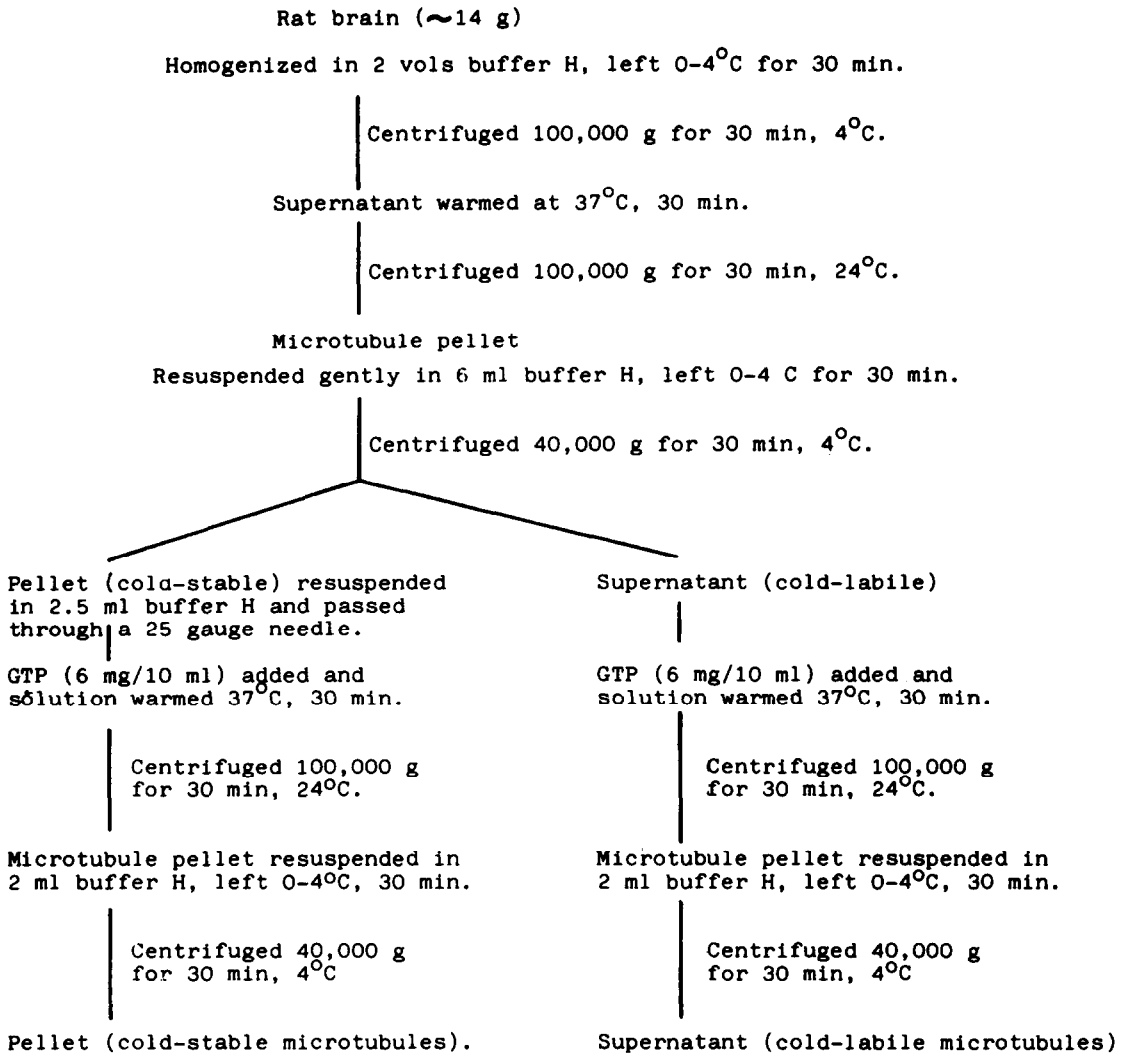
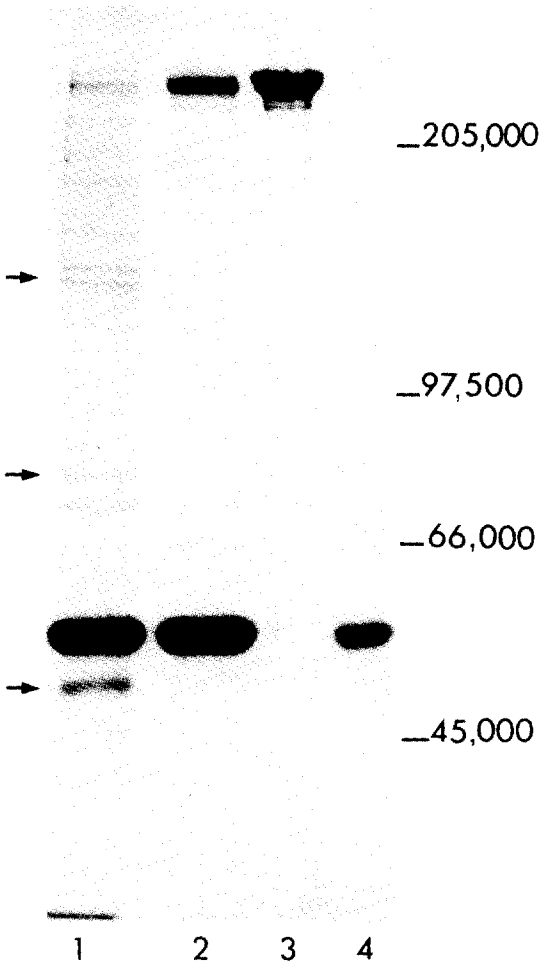


Fig.1. Preparation of cold-stable and cold-labile microtubules.

MAP 1 component was similar in both cold-stable (8% ± 1%) and cold-labile (6% ± 1%) preparations. In contrast the cold-stable microtubules were enriched in 4 minor components, two of approx. 155 kDa, one of 80 kDa and one of 50 kDa.

Both cold-stable and cold-labile preparations exhibited alkaline phosphatase activity at pH 10.4 although the activity in the cold-labile preparations was very low. As shown in table 1, the specific activity was much greater (~7 fold) in the cold-stable compared to the cold-labile microtubules. Activity in both types of preparation was stimulated by either 5 mM Mg^{2+} or 0.5 mM Zn^{2+} and the extent of stimulation was similar regardless of susceptibility to cold. In both preparations the stimulation was greater by zinc (0.5 mM) than by Mg^{2+} (5 mM).

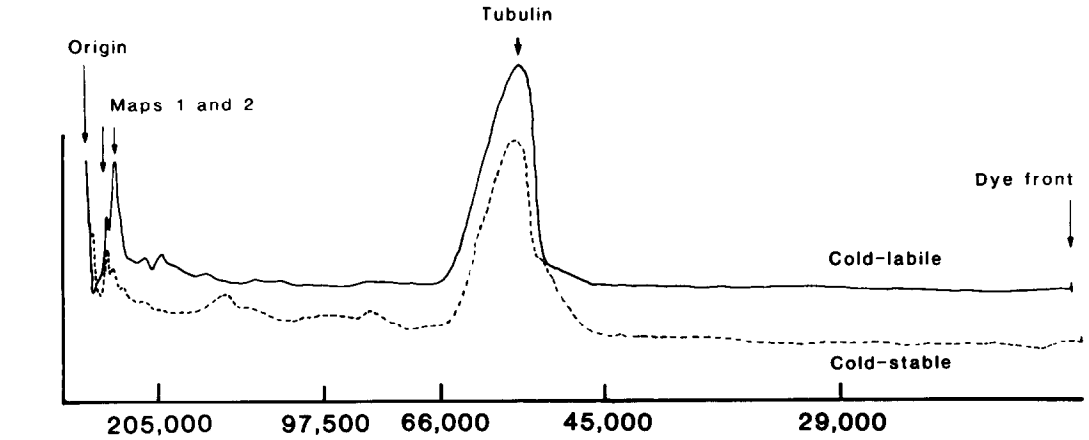
Stimulation of microtubule assembly by zinc was found to be associated with an increase in alkaline phosphatase specific activity (table 2). Dialysis of microtubules once the tubules were formed did not destroy the effect on alkaline phosphatase activity. In contrast, dialysis of microtubules exposed to zinc after assembly drastically reduced the alkaline phosphatase activity. It would



thus appear that zinc added to microtubules after assembly is readily removed by brief dialysis whereas the presence of zinc during assembly leads to a stronger association of zinc with microtubules such that it is not removed by brief dialysis. The increase in alkaline phosphatase activity found in microtubules assembled in the presence of zinc is therefore probably an effect of zinc bound, relatively tightly, to microtubules. Mg^{2+} appears to be more strongly bound than zinc when added to assembled microtubules since dialysis had little effect on the alkaline phosphatase activity.

4. DISCUSSION

This study presents evidence for there being significant differences in the polypeptide composition and enzyme activity between cold-stable and cold-labile microtubules. Firstly, cold-labile microtubular protein was found to contain a greater proportion of the high molecular mass MAP 2, and secondly cold-stable microtubules were enriched in certain minor components, of approx. 155, 80 and 50 kDa; the latter components probably correspond to cold-stable specific proteins observed previously [8,9]. Earlier studies of cold-stable and cold-lable microtubules failed to show any difference in the high molecular mass MAPs or other components [5,6], however, the strategies used in these earlier studies may have led to incomplete



b

Fig.2. (a) SDS-gel electrophoresis of cold-stable and cold-labile microtubules: Lane 1, cold-stable microtubules; lane 2, cold-labile microtubules; lane 3, heat-stable supernatant prepared by heating twice cycled microtubule protein for 5 min at 100°C followed by centrifugation; mostly MAP 2. Lane 4, tubulin. Arrows show position of possible cold-stable specific proteins. Migration positions of known standards (myosin, phosphorylase *b*, bovine serum albumin and ovalbumin) are shown at the side. (b) Densitometric scan of Coomassie blue-stained gels of cold-stable and cold-labile microtubules.

Table 1

Alkaline phosphatase activity in cold-stable and cold-labile microtubules

Added cations	Specific activity (nmol *p*-nitrophenol liberated/ hr per mg protein)		Activity as % of that in the absence of added cations	
	Cold-stable	Cold-labile	Cold-stable	Cold-labile
None	147 ± 28 (7)	19 ± 5 (9)	–	–
5 mM Mg^{2+}	224 ± 44 (4)	51 ± 14 (4)	213 ± 39 (4)	271 ± 87 (4)
0.5 mM Zn^{2+}	335 ± 37 (5)	47 ± 14 (5)	302 ± 73 (5)	327 ± 87 (5)

Values shown are means ± SE with the number of preparations studied in parentheses

Table 2

Effects of cations and of stimulation of rat brain microtubule assembly by zinc on microtubule alkaline phosphatase activity

Assembly conditions	Cations added to assembled microtubules	Specific activity (nmol *p*-nitrophenol liberated/h per mg protein)	
		Samples not dialysed	Samples dialysed
Control	none	54 ± 7 (6)	56 ± 3 (6)
Control	5 mM Mg^{2+}	135 ± 35 (4)	126 ± 26 (4)
Control	500 μM Zn^{2+}	173 ± 28 (4)	78 ± 26 (4)
+500 μM Zn^{2+}	none	100 ± 5 (6)	102 ± 7 (4)

Values given are means ± SE with the number of preparations studied in parentheses

separation of the cold-labile microtubules in the final step of purification and since cold-labile microtubules, upon warming, repolymerize to form microtubules of mixed stability [8], this may have led to a certain proportion of stable microtubules in the so-called cold-labile material. The use of exposure to both cold and calcium also complicated the situation in one study [5]. Cold-stable and cold-labile microtubules prepared by methods similar to those used here [8,9] showed differences in minor components but not in the high molecular mass MAPs. It is difficult to explain this discrepancy; however, it is possible that a differnce in the high molecular mass MAPs was not seen previously due to overloading of the gels in order to see minor cold-stable specific components [8,9]. The present results suggest that brain microtubules which are labile to cold contain a higher proportion of MAP 2 than those that are cold-stable. This, in turn, suggests that various classes of microtubules may exist, differing in their stability. There is some evidence that the brain contains several types of microtubules [16] and MAP 2 has been shown to be preferentially associated with dendritic microtubules [17]. It may be that dendritic microtubules are an example of cold-labile microtubules.

In addition to the differences in polypeptide composition, cold-stable and cold-labile microtubules were found to differ in their alkaline phosphatase specific activity, the cold-stable microtubules exhibiting a much greater activity of the enzyme. The microtubule-associated alkaline phosphatase activity is poorly characterized and its function unknown; it is thought however to be associated with the associated proteins, possibly

MAP 1, rather than tubulin [12,18]. Its involvement in protein phosphorylation reactions [19] or ATPase activity has not been defined and it is difficult therefore to assess the full significance of the difference in phosphatase activity associated with cold stability. If the activity is that of a protein phosphatase, the observed increase in activity might cause a reduced phosphorylation of some microtubule component in cold-stable microtubules. This is consistent with the suggestion [9] that cold-lability is associated with increased phosphorylation of certain microtubule proteins; alternatively cold-lability might be associated with phosphorylation of MAP 2. MAP 2 is known to be phosphorylated and this phosphorylation has been shown to aeffect microtubule assembly [20].

The addition of zinc to rat brain extracts stimulates microtubule assembly [10,11] and stimulation of assembly by zinc was found (table 2) to cause an increase in alkaline phosphatase specific activity. Dialysis experiments suggest that this is due to binding of zinc to microtubule protein during assembly; this binding appears to be different from that which occurs on incubation of assembled microtubules with zinc. Thus the results suggest both that stimulation of assembly by zinc [10,11] is associated with a binding of zinc to microtubules and that microtubule assembly leads to changes in Zn-binding properties of the constituent microtubule proteins. The concomitant increase in both alkaline phosphatase activity and cold-stability [11] in response to changes in assembly is further evidence of an association between cold-stability and alkaline phosphatase activity. It remains to be investigated whether this is due to the alkaline phosphatase being involved in the regulatory mechanism of microtubule stability.

In conclusion, the present results show 3 differences between cold-stable and cold-labile microtubules. How, if at all, these differences are related, is not known. They are, however, 3 factors which may be involved in the regulation of microtubule stability.

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