LYSOZYME-INDUCED POLYMERIZATION OF TUBULIN

Burial of the colchicine-binding site as a probe

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1. Introduction

Ample evidence has accumulated indicating that the information for forming microtubule lattice lies in the tubulin molecule. There are varieties of agents such as Mg^{2+} [1–3], dimethyl sulfoxide [4], polyethylene glycol, DEAE-dextran [5] and basic proteins [6–9] which could induce microtubule assembly in a purified preparation of tubulin. However, it appears that in those assembly conditions where polycations are employed, tubulin is marginally soluble and microtubule assembly occurs just before precipitation [5,10]. Polycation-induced assembly can not be reversed in all cases even by prolonged cooling [6,9], whereas, normal assembly (without adding exogenous factor) is quite susceptible to cold, Ca^{2+} and some antimitotic drugs. Although turbidity is a reliable measure of the assembly of tubulin into larger forms, only a reversible turbidity (upon cooling the sample) could indicate the microtubule assembly [11]. Therefore, to follow the microtubule assembly even in the presence of non-specific aggregation (which is unavoidable in the presence of lysozyme), we have explored the use of colchicine-binding as a probe. We now report experiments demonstrating the burial of the colchicine-binding site of tubulin as assembly occurs and also the sensitivity of the lysozyme-induced tubulin polymers toward Ca^{2+} and cold, as observed using colchicine-binding as a probe.

2. Materials and methods

Tubulin was purified from goat brain according to [12] except that DEAE-cellulose (Whatman DE 52) was used instead of DEAE–Sephadex A-50 [13] in PMG buffer [10 mM phosphate (pH 7.0), 10 mM $MgCl_2$ and 0.1 mM GTP]. The active fractions as judged from colchicine-binding assay [14,15] were pooled, concentrated by overnight dialysis at 0°C against 8 M glycerol in either MES [2(*N*-morpholino)ethanesulfonic acid] buffer [100 mM MES (pH 6.4), 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP], or PMG buffer depending on the nature of subsequent experiments and stored at –70°C. The purity of the protein was checked by SDS–polyacrylamide gel electrophoresis according to [16].

Tubulin was allowed to polymerize at 37°C either in buffer A, [100 mM MES (pH 6.4), 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP and 4 M glycerol] which was used for normal assembly [17,18], or in buffer B [10 mM phosphate (pH 7.0), 10 mM $MgCl_2$, 0.1 mM GTP and 3.4 M glycerol] which was used for lysozyme-induced assembly. It should be noted that although DEAE-purified tubulin could be polymerized without any added factor(s) in buffer A [19], no microtubule formation could be detected in buffer B unless lysozyme was added. In both cases polymerization was monitored turbidometrically [11]. Electron microscopy was done as in [20]. A 10 μl microtubule sample was placed for 2–5 min on a collodion-coated copper grid (400 mesh) and displaced successively with 4 drops of each of distilled water and 1% uranyl acetate. The excess stain was removed with a filter paper. Grids were examined with a Siemens Elmiskop 101B electron microscope at an accelerating voltage of 80 kV.

3. Results and discussion

To determine whether the colchicine-binding site of tubulin is blocked during assembly process, tubulin was incubated either alone in buffer A (fig.1A) or with

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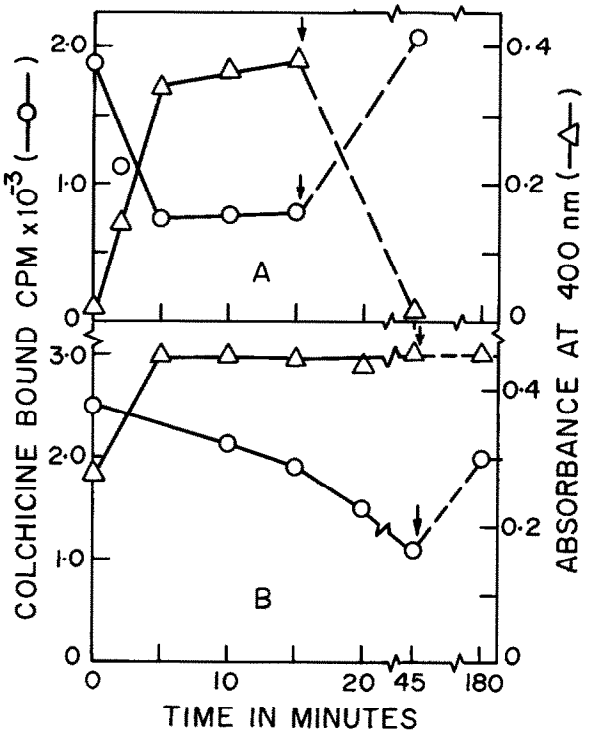

Fig.1. Colchicine-binding activity during polymerization of tubulin. (A) Tubulin (2.0 mg/ml) in buffer A was incubated at 37°C and polymerization was monitored turbidometrically at 400 nm (△). At the time indicated by arrow the tube was placed at 0°C and incubated there. At different time intervals, aliquots were withdrawn and immediately incubated with [^{3}H]colchicine (1 × 10^{-5} M) at 37°C for 10 min to determine the colchicine-binding activity (○). (B) Tubulin (1.6 mg/ml) was incubated with lysozyme (0.8 mg/ml) in buffer B at 37°C and the turbidity at 400 nm (△) was measured at different time intervals. At the time indicated by the arrow the tube was placed at 0°C and incubated there. At different time intervals aliquots were withdrawn and incubated immediately with [^{3}H]colchicine (1 × 10^{-5} M) at 37°C for 10 min to determine the colchicine-binding activity (○).

lysozyme in buffer B (fig.1B); aliquots were taken out from the polymerizing mixtures at the indicated periods and colchicine-binding activities were determined. The results reveal that as polymerization was in progress, evident from rise in turbidity, the colchicine-binding activity declined, in normal as well as in lysozyme-induced assembly. To test the temperature sensitivity, these polymerized mixtures were then kept at 0°C for 30 min; in the case of normal polymerization the turbidity and also the colchicine-binding activity returned to their original levels. In the case of lysozyme-induced polymerization, the turbidity remained unaltered upon such cooling. This apparently indicated that unlike normal assembly, the lysozyme-induced microtubule assembly was perhaps insensitive to cold. But a totally different picture emerged when colchicine-binding was measured; upon cooling the polymerized mixture, the colchicine-binding activity returned almost to the original level, and in the case of lysozyme-induced assembly (fig.1B). This result indicates that the turbidometric assay may not be sensitive enough to measure the polycation-induced assembly, where most of the turbidity is generated by aggregation and precipitations of protein; thus the contribution of microtubules towards the total turbidity can not be ascertained. These discrepancies may arise because turbidity as an assay method is based on the mass of any high molecular weight material, whereas colchicine-binding to tubulin can monitor a change in the tubulin molecule itself, occurring during the polymerization process.

Electron microscopic examination has revealed a striking morphological difference between the two types of polymerized tubulin as depicted in fig.2. Although quite normal microtubules are assembled from tubulin in buffer A (fig.2A) it is interesting to find that the lysozyme-induced polymerization of tubulin in buffer B leads to the formation of polymers of very different morphology (fig.2B). However, this structure disappeared on cooling at 4°C (not shown) whereas amorphous materials remain, even on prolonged cooling. The presence of amorphous material in the preparation containing lysozyme has probably arisen from non-specific aggregation.

The lysozyme-induced assembly was further tested with respect to Ca^{2+}-sensitivity. Here, tubulin was incubated with different concentrations of lysozyme in buffer B at 37°C for 30 min and colchicine-binding activities of the samples were determined. As shown in fig.3A, the colchicine-binding activity declined with increasing lysozyme concentration. However, such inhibition of colchicine-binding activity due to lysozyme-induced assembly could be reversed upon treatment of the incubated samples with Ca^{2+} (4 × 10^{-3} M) or cold (0°C for 3 h) prior to the addition of [^{3}H]colchicine. Thus, it appeared that both normal and lysozyme-induced microtubules behave similarly with respect to colchicine-binding. Additional evidence in favour of such Ca^{2+}-sensitivity emerged also from the experiments where tubulin had been pretreated with Ca^{2+} before the addition of lysozyme. The results in fig.3B indicate that pretreatment of tubulin with 3 × 10^{-5} M

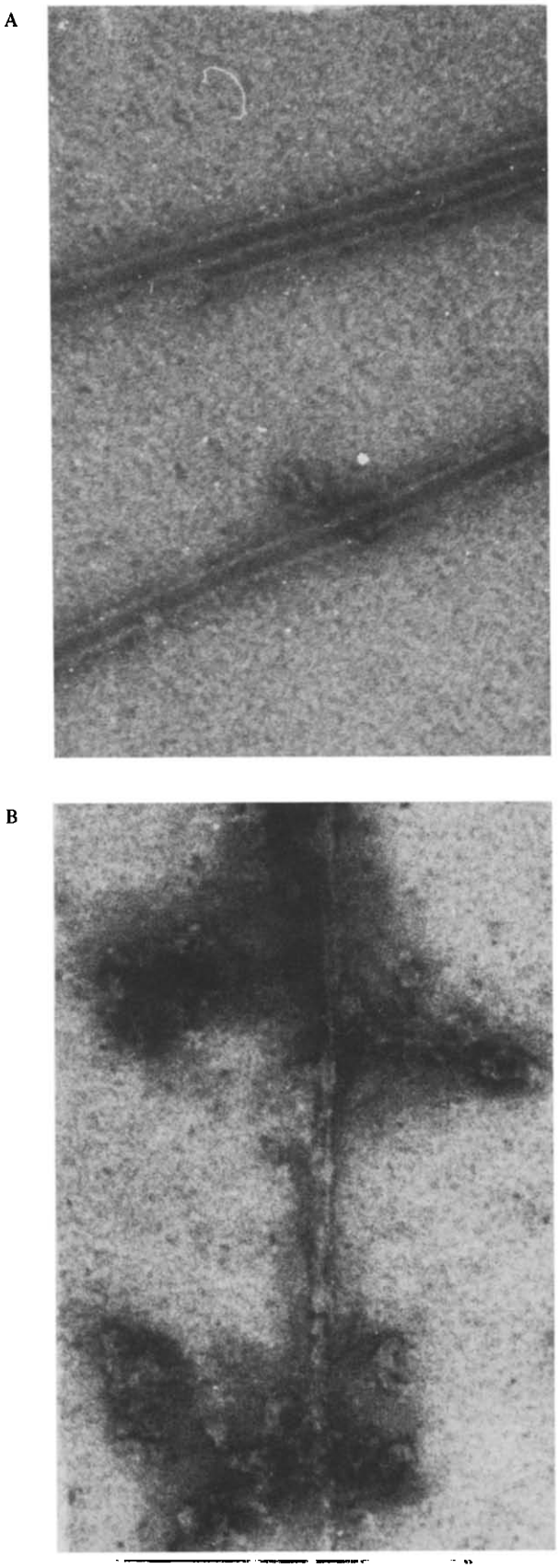

Fig.2. Electron micrographs of 1% uranyl acetate negatively stained polymers obtained from DEAE-purified tubulin in absence (A) and in presence (B) of lysozyme. (A) Normal microtubules assembled from tubulin (2.0 mg/ml) in buffer A. Magnifications: 200 000 ×. (B) Abnormal polymers formed from tubulin (2.0 mg/ml) in presence of lysozyme (2.0 mg/ml) in buffer B. Magnifications: 200 000 ×.

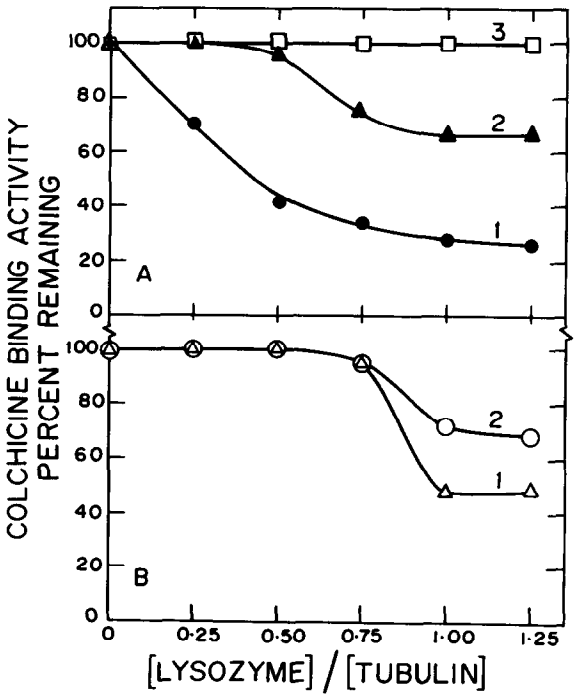

Fig.3. Effect of Ca^{2+} and cold on lysozyme-induced inhibition of colchicine-binding to tubulin. (A) Aliquots of tubulin (1.6 mg/ml) in buffer B were incubated with increasing concentrations of lysozyme at 37°C for 30 min. After the incubation period, colchicine-binding activities of the samples were determined: immediately (1); after post-incubation at 0°C for 3 h (2); after post-incubation with 4 mM $CaCl_2$ at 37°C for 30 min (3). (B) Aliquots of tubulin (1.6 mg/ml) in buffer B were pretreated with Ca^{2+} (3×10^{-5} M in (1) and 1.25×10^{-4}M in (2)) at 37°C for 10 min, then incubated with increasing concentrations of lysozyme at 37°C for 30 min as in the case of (A). After the incubation period, the colchicine-binding activities of the samples were determined immediately. In all cases (A,B), colchicine-binding activities were measured by incubating the samples with [^{3}H]colchicine (1×10^{-6} M) at 37°C for 30 min and data are expressed as % total binding obtained with tubulin in the absence of lysozyme.

Ca^{2+} could prevent the inhibition of colchicine-binding when the lysozyme:tubulin ratio was 0.75. However, at a higher lysozyme:tubulin ratio, a higher [Ca^{2+}] was required to withdraw the inhibition of colchicine binding. Nevertheless, in the case of lysozyme-induced assembly, pretreatment with Ca^{2+} could not prevent the precipitation of protein as observed by turbidometry (not shown).

We may conclude that both in normal and in lysozyme-induced microtubules, the colchicine-binding sites are buried. Pretreatment of tubulin with Ca^{2+} prevents microtubule formation both in normal and lysozyme-induced assembly. Furthermore, both Ca^{2+} and cold depolymerize both types of microtubules with simultaneous exposure of the colchicine-binding site. Electron microscopic studies have shown a novel feature of microtubule structure in the case of lysozyme-induced assembly.

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