CALCIUM AFFINITY OF CHICK BRAIN TUBULIN

Ilona Staprans, William C. Kenney* and Ellen Roter Dirksen

Cancer Research Institute
University of California, San Francisco, California 94143

and

*Molecular Biology Division
Veterans Administration Hospital
San Francisco, California 94121

and

Department of Biochemistry and Biophysics
University of California, San Francisco, California 94143

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We have found that during polymerization tubulin displays a large increase in intrinsic tryptophan fluorescence. This change in observed fluorescence was utilized to study the effect of calcium on the polymerization of crude and purified chick brain tubulin. No difference in calcium sensitivity was observed when tubulin was polymerized either in crude extracts or purified preparations, indicating that the possibility of a separate regulatory factor could be eliminated. Quantitative calcium binding measurements show that tubulin has no significant affinity for calcium as compared to the well known calcium-binding protein, troponin C.

INTRODUCTION

There is recent experimental evidence that calcium is a strong inhibitor of the polymerization of brain tubulin (1,2) and that assembled cytoplasmic microtubules can be depolymerized by addition of external calcium (3). Since calcium has a regulatory role in a wide variety of biological functions, tubulin sensitivity to calcium suggests that the tubulin-microtubule equilibrium may be regulated by calcium concentrations. At present, no evidence has been found to indicate the presence of a regulatory factor which could establish an intracellular calcium gradient.

We have utilized a change in the intrinsic fluorescence of tubulin as an assay to study the effect of calcium on the rate of assembly of this protein isolated from chick brain. A comparison of crude, partially purified and purified tubulin preparations might establish the presence of a factor which

could affect calcium sensitivity during tubulin assembly. To further elucidate the calcium effect during polymerization, we also studied quantitatively the extent to which tubulin binds calcium. We compared these results to the data obtained for well characterized calcium-binding substances such as calcium chelator EGTA* and the calcium-binding subunit of rabbit muscle troponin (troponin C).

MATERIALS AND METHODS

Tubulin was prepared from 2 day-old chick brains by the method of Shelanski et al (4) and purified either through one or two cycles of polymerization, while troponin C from rabbit skeletal muscle was prepared as described by Staprans et al (5). Protein concentrations were determined by the method of Lowry et al (6).

Fluorescence measurements were made with a Perkin-Elmer Model MFP-3 spectrofluorometer in which the sample compartment was water-jacketed to maintain a constant temperature of 37°C. Tryptophan fluorescence of microtubule protein was observed at 345 nm after excitation at 232 nm. Experimental data were obtained by following the increase in fluorescence as a function of incubation time. 0.1 M MES† buffer (pH 6.5) containing 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP was used during all fluorescence measurements. The free calcium concentration was adjusted to account for the EGTA present in the buffer system. To obtain results which were internally comparable, the same calcium and EGTA stock solutions were used for all experiments.

The chelex-100 method of Briggs and Fleishman (7) was used to determine the presence of calcium-binding substance. This assay depends on the differential competition for ^{45}Ca between chelex-100 and calcium complexing material. Chelex was pipetted into tubes to a final sedimented volume of 0.2 ml. A solution containing the calcium-binding substance was added, and the final volume was adjusted to 5.0 ml. 30 mM imidazole buffer (pH 7) containing 4 mM $MgCl_2$

*ethylene glycol bis(B-aminoethylester)-N,N-tetraacetic acid.
†2-(N-morpholino) ethane sulfonic acid.

and 50 mM KCl was used for the binding measurements. The final mixture was stirred for 15 sec, the resin sedimented and a 1 ml sample was removed from the supernatant for radioactivity measurement in a Packard liquid scintillation counter. By comparing the binding properties of tubulin to those of EGTA and troponin C, the approximate binding constant of tubulin could be determined.

RESULTS AND DISCUSSION

The increase in viscosity during the assembly of brain tubulin has been well documented (2,3). We have found similarly that when tubulin, prepared at 0°C, is incubated at 37°C, there is a rapid increase in tryptophan fluorescence which reaches a plateau within 10 min (Fig. 1). The sharp drop of measured fluorescence of assembled microtubules to the original level when the temperature is lowered to 0°C and the lack of increase in fluorescence measurements when 5.0 x 10^{-5}M colchichine is added to the incubation mixture (Fig. 1) correlates with the observation that microtubule assembly is reversed by low

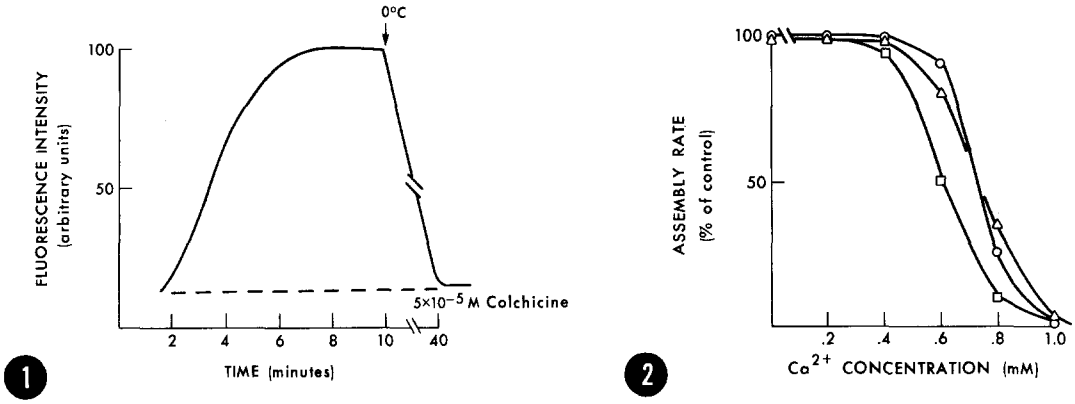


Figure 1. Effect of temperature and colchicine on tubulin polymerization as a function of fluorescence (arbitrary units). Protein concentration, 1.0 mg/ml. Tubulin was incubated at 37°C as described in METHODS. At the arrow the incubation mixture was taken to 0°C. Dashed line represents fluorescence in the presence of 5.0 x 10^{-5}M colchicine.

Figure 2. Effect of Ca^{2+} ions on the rate of polymerization of original chick brain extract, 5.0 mg/ml, (□). One cycle of polymerization of tubulin, 1.0 mg/ml (○). Two cycles of polymerization of tubulin, 1.0 mg/ml (△). The rate of polymerization is inversely proportional to the time required to reach the maximum level.

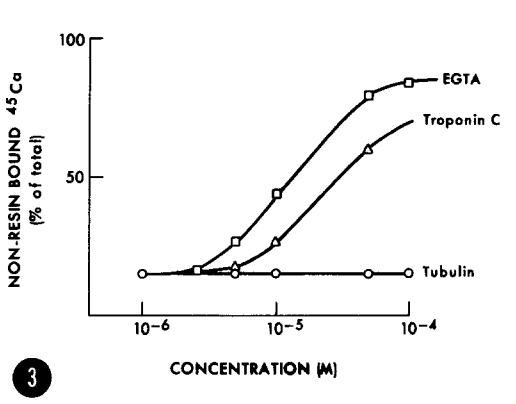
temperature and inhibited by colchicine (8). These results indicate that a large conformational change, associated with polymerization, has taken place. This increased fluorescence, which reflects the biological properties of microtubule assembly, provides a more sensitive assay for the study of assembly than viscosity measurements, particularly at very low protein concentrations. It should be noted that there was a measurable but insignificant change in fluorescence during polymerization when the excitation wavelength was 280 nm, which was possibly due to scattering.

Fig. 2 demonstrates the effect of calcium on the rate of polymerization of chick brain tubulin. Tubulin solutions at different stages of purification (crude extract and after 1 and 2 cycles of polymerization) were adjusted to varying Ca^{2+} ion concentrations in EGTA-containing buffer before incubation at 37°C and the fluorescence followed. Since in these experiments calcium has no effect on the final plateau reached, but only on the time required to reach the plateau, the data have been expressed as changes in fluorescence per minute of incubation time. No significant difference in calcium sensitivity was observed among the crude and purified preparations. In the presence of 1 mM calcium the rate of change in fluorescence was too low to measure, which indicates that complete inhibition of assembly had essentially occurred in all preparations studied. It should be noted that this inhibition can be reversed by addition of 1 mM EGTA. Considering these results, we conclude that it is very unlikely that crude tubulin homogenates contain a separate factor which is responsible for calcium sensitivity during microtubule assembly.

The effective calcium inhibition range in our measurements is in agreement with the results obtained by Olmsted and Borisy (2) who used viscosity as a measure of polymerization. However, differing from our observations that calcium affects only the rate of polymerization, their viscosity measurements show that calcium also depresses the final plateau level attained. Since fluorescence measures the conformational change of protein and viscosity increase measures the formation of long polymers, these combined results indi-

cate that calcium affects, not only the rate of polymerization, but also the length of microtubules formed. However, at present there are no quantitative results which could correlate the length of microtubules with fluorescence measurements.

The calcium effect on tubulin assembly was further examined in binding experiments to determine the extent to which tubulin binds exchangeable calcium. When the total amount of calcium is held constant at 10^{-5}M, there is a rise in non-resin bound ^{45}Ca with increasing EGTA or troponin C concentrations (Fig. 3). The concentration range over which the non-resin bound ^{45}Ca increases is dependent on the calcium-binding constant of the calcium-sequestering agent present. Experimentally, we have assumed that the concentration at which only 50% of the counts were bound to the resin was inversely proportional to the binding constant, $1/C \propto K$, where $\underline{C}$ is the concentration required to bind 50% of the available counts and $\underline{K}$ is the binding constant. For the conditions used in these experiments, the binding constant for EGTA was assumed to be



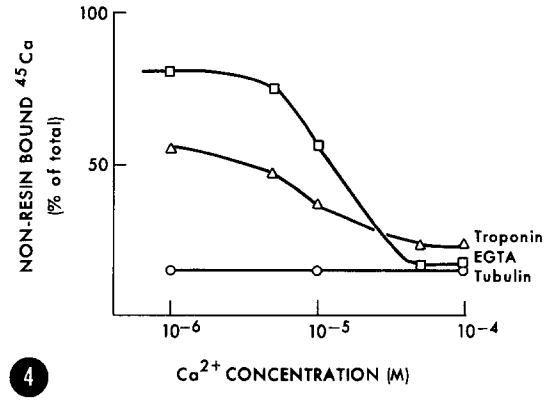


Figure 3. Effect of concentrations of EGTA (□), troponin C (△) and tubulin (○) on the binding of ^{45}Ca to chelex-100. Final Ca^{2+} ion concentration is 10^{-5}M. Tubulin was purified through two cycles of assembly and then dizlyzed extensively to remove any EGTA present during the preparation procedure.

Figure 4. Effect of calcium concentration on the binding of ^{45}Ca to chelex-100 in the presence of EGTA (□), troponin C (△) and tubulin (○). EGTA was added to a final concentration of 2×10^{-5} M. Protein concentration for troponin C was 1 mg/ml and for tubulin, 2.5 mg/ml. Tubulin was prepared as explained in the legend for Fig. 3.

10^7 (9). Using the concentrations obtained for 50% binding from Fig. 3, the approximate binding constant for troponin and tubulin could be calculated with the use of the following relationship: $(1/C) / (1/C_{EGTA}) = K/K_{EGTA}$. The K for troponin was estimated to be 10^6, which is in agreement with the values in the literature (10). For tubulin, however, there was no evidence for any calcium binding since the percentage of non-resin bound ^{45}Ca remained constant, even at relatively high tubulin concentrations (5 mg/ml).

In those experiments where the calcium concentration was varied, there was also no detectable calcium binding (Fig. 4). For EGTA and troponin C, Ca^{2+} ion distribution was affected by a change in calcium concentration, but no binding occurred for tubulin even at low calcium concentrations. These results indicate that tubulin has a very low affinity for calcium and that our tubulin preparations show no indication of significant calcium binding. At present, the physiological relevance of brain tubulin sensitivity to calcium remains unanswered, since there is no explanation for the calcium sensitivity tubulin displays during polymerization.

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