CALCIUM BINDING TO BOVINE BRAIN TUBULIN

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1. Introduction

Microtubules are present in a wide variety of eucaryotic cells and it was recently shown that the reassembly in vitro of brain microtubular protein (tubulin) can be reversibly controlled by calcium ions [1–6]. Calcium plays a regulatory role in a wide variety of biological functions. However, little is known regarding the interactions between calcium and tubulin, or the extent to which such interactions might play a role in mediating the effects of calcium on cell function.

In this report, we present evidence to show that calcium ions are preferentially bound to the disassembled form of tubulin.

2. Experimental

2.1. *Preparation of tubulin*

Bovine brain was minced and homogenized with 1.2 vols of the reassembly buffer of Kuriyama (5 mM MES*, 50 mM KCl, 1 mM ATP, 0.5 mM $MgCl_2$ and 1 mM EGTA (pH 6.6)) [7] and tubulin was purified by the reassembly–disassembly method of Shelanski et al. [3]. Purified disassembled tubulin was finally suspended in 8 M glycerol-reassembly buffer and stocked at −20°C. Immediately prior to use, an aliquot of the stock solution of tubulin was chromatographed on Sephadex G-25 (coarse), which had been equilibrated at 4°C with elution buffer containing 5 mM MES, 50 mM KCl, 1 mM ATP and 0.5 mM $MgCl_2$ (pH 6.6), in order to remove glycerol and EGTA. Tubulin recovered in the void volume was reassembled at 37°C for 30 min in the absence of EGTA and glycerol as reported by Olmsted and Borisy [2], and the sediment obtained after centrifugation at 59 000 *g* for 30 min at 30°C was resuspended at 37°C in an appropriate volume of the elution buffer to give a favorable concentration of protein of about 4–8 mg/ml for calcium binding assay. To get disassembled tubulin, tubulin reassembled in the elution buffer was immersed in an ice-bath for 30 min, then centrifuged at 105 000 *g* for 60 min at 4°C. About 80% of protein was solubilized under these condition. The reassembly and disassembly of tubulin were monitored turbidimetrically [6] and confirmed by electron microscopy in which specimens were negatively stained with 1% uranyl acetate and examined in a JEM 100 C electron microscope.

2.2. *Calcium binding assay*

The binding of calcium to tubulin was measured by a gel filtration method, using $^{45}Ca^{2+}$. One ml aliquots of a tubulin preparation (2–7 mg/ml) containing 0.01 mM $^{45}CaCl_2$ were chromatographed at 4°C, for disassembled tubulin or at 37°C, for reassembled tubulin, on a Sephadex G-25 column (0.9 cm × 28 cm) which had been equilibrated with elution buffer (5 mM MES, 50 mM KCl, 1 mM ATP and 0.5 mM $MgCl_2$ (pH 6.6)) containing 0.01 mM $^{45}CaCl_2$ (0.02 μCi/ml). 16 drop fractions (about 0.4 ml) were collected at a flow rate of 25 ml/h.

*Abbreviations: MES, 2-(*N*-morpholino)-ethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl-ether)-*N,N'*-tetraacetic acid.

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The protein concentration in each fraction was determined according to the method of Lowry et al. [8]. No interference with solvent used was observed. The $^{45}Ca^{2+}$ radioactivity was measured with a liquid scintillation spectrometer, Aloka model LSC-651.

2.3. *SDS-polyacrylamide gel electrophoresis*

SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn [9] in 7.5% polyacrylamide gels and proteins were stained with Coomassie blue. The stained gels were scanned on a Gilford 250 recording spectrophotometer, fitted with a gel scanner of model 2410-S linear transport.

3. Results

Fig.1 shows an electron micrograph and an SDS-polyacrylamide gel electrophoresis pattern of a typical preparation of tubulin used in this report. The structure of reassembled tubulin in 0.01 mM $CaCl_2$ was not different from that seen in the presence of 1 mM EGTA by electron microscopy. The purity of the tubulin was estimated to be about 82% by densitometry of stained SDS-polyacrylamide gels. High molecular weight proteins accounted for about 15% of the stained protein, as was reported by Borisy et al. [10].

To measure the binding of calcium to tubulin, Sephadex G-25 (coarse) equilibrated with the elution buffer containing 0.01 mM $^{45}CaCl_2$ was used.

Fig.1. An electron micrograph (× 20 000) and an SDS-polyacrylamide gel electrophoresis pattern of reassembled tubulin.

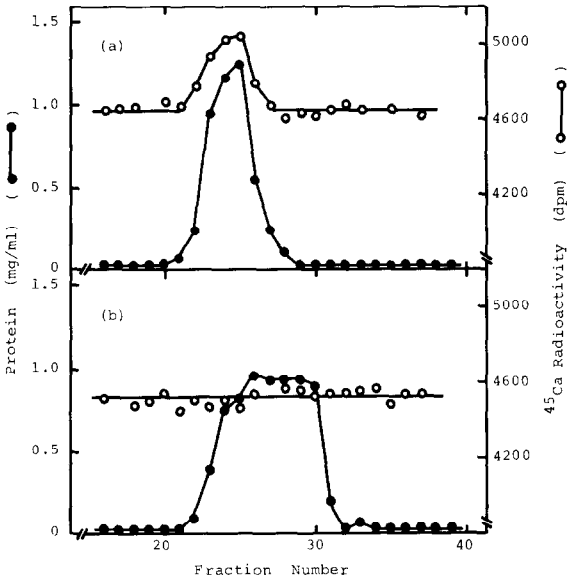

Fig.2. Calcium binding of (a) disassembled tubulin at 4°C (b) reassembled tubulin at 37°C. One ml of disassembled tubulin (3.8 mg/ml) in 5 mM MES, 50 mM KCl, 1 mM ATP and 0.5 mM $MgCl_2$ (pH 6.6) was applied on Sephadex G-25 column (0.9 cm × 28 cm) equilibrated with the above solution containing 0.01 mM $^{45}CaCl_2$ (0.02 μCi/ml) at 4°C. In reassembled tubulin (4.6 mg/ml), the same procedure was performed at 37°C.

Fig.2(a) shows that disassembled tubulin bound calcium at 4°C. On the other hand, reassembled tubulin chromatographed at 37°C did not bind calcium (fig.2(b)). The addition of 0.01 mM $^{45}CaCl_2$ before disassembly and reassembly did not change the amount of $^{45}Ca^{2+}$ bound to disassembled tubulin and reassembled tubulin, respectively. These results suggest that the bound calcium is released from tubulin during reassembly. The amount of calcium bound to tubulin was independent of a cycle of disassembly–reassembly.

Disassembled tubulin is known to be a mixture of a 6 S dimer and a 36 S component [11]. In order to know whether the 6 S dimer or the 36 S component binds calcium, the gel filtration method was used. Sephadex G-200 column chromatography of 2 ml of disassembled tubulin (6.5 mg/ml) eluted at 4°C with 4 M glycerol-reassembly buffer showed two peaks of protein corresponding to the 36 S component and the 6 S dimer (fig.3) [7]. SDS-poly-

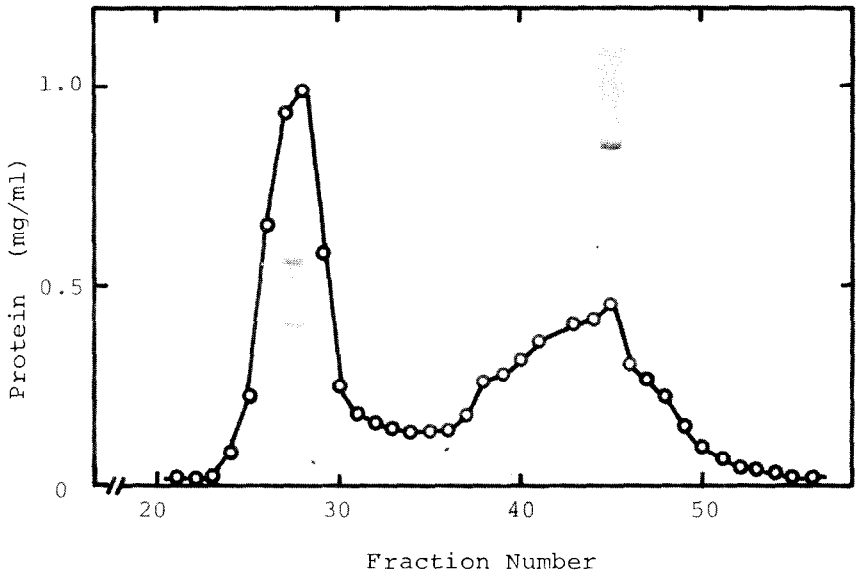

Fig.3. Separation of 36 S component and 6 S dimer tubulin on Sephadex G 200 column chromatography (1.4 cm X 48 cm). 2 ml of disassembled tubulin (6.5 mg/ml) in 4 M glycerol-reassembly buffer was applied and eluted with the same solvent at 4°C. Each 1.2 ml was collected at flow rate of 7.5 ml/cm²/h.

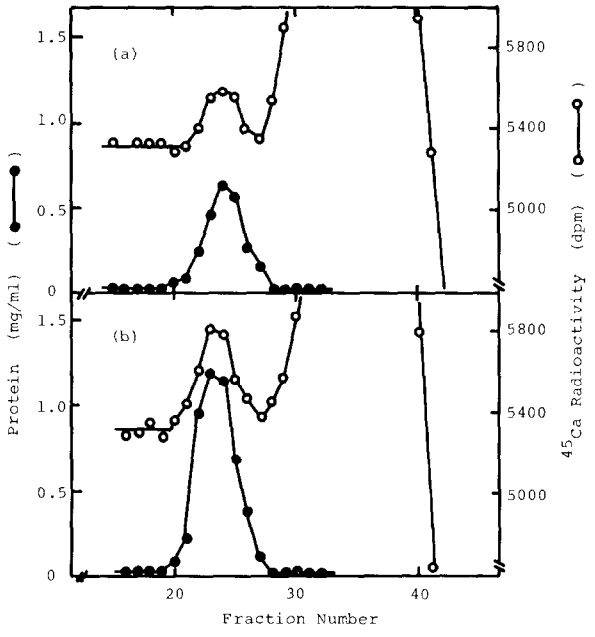

Fig.4. Calcium binding of (a) 36 S component and (b) 6 S dimer tubulin at 4°C. The procedure was the same as in fig.2.

acrylamide gel electrophoresis indicated no contamination of high molecular weight proteins in the 6 S dimer fractions. One ml of 36 S component and 6 S dimer was separately applied on a Sephadex G-25 column equilibrated with the elution buffer containing 0.01 mM $^{45}CaCl_2$ (0.02 μCi/ml at 4°C for measurements of calcium binding. The results showed that both the 36 S component and the 6 S dimer bound calcium to about the same extent (fig.4). The large calcium binding peak seen after the protein peak is due to the presence of EGTA in the original tubulin solvent.

4. Discussion

Our comparative study of calcium binding showed that disassembled tubulin bound calcium at 4°C, but reassembled tubulin did not bind calcium at 37°C in the same solvent. This suggests that the tubulin-microtubule equilibrium is directly regulated by the calcium concentration.

Recently, Staprans et al. [12] failed to demon-

strate the binding of calcium to chick brain tubulin with the Chelex-100 method, in which free Ca^{2+} was removed by a calcium chelating resin, Chelex-100, and the calcium remaining complexed with calcium binding protein was measured. In our earlier experiments, we tried to measure calcium binding with a nitro-cellulose membrane method, in which a mixture of protein and calcium was passed through a nitro-cellulose membrane, free Ca^{2+} was washed away with elution buffer, then calcium bound to the protein deposited on the membrane was measured. But we had failed to demonstrate the binding of calcium to bovine brain tubulin with the nitro-cellulose membrane method, although the same procedure was used to measure the calcium binding activity of plasmodium calcium binding protein [13]. This suggests that the equilibrium rate of Ca^{2+} + tubulin $\rightleftarrows$ Ca-tubulin may be too fast to allow the detection of Ca-tubulin after removal of free Ca^{2+}.

The molar ratio of bound calcium to tubulin dimer was about 0.1 using an elution buffer containing 0.01 mM $CaCl_2$. The low value of this ratio may be due to the low concentration of free Ca^{2+} in the solution. The comparison of calcium binding between reassembled and disassembled tubulin in the same solvent did not allow the use of higher $CaCl_2$ concentration which might bring about disassembly of reassembled tubulin. Free Ca^{2+} concentration in the tubulin fractions which did not contain EGTA in figures 2 and 4 was estimated as 0.0012 mM in the elution buffer containing 0.01 mM $CaCl_2$ from the dissociation constant of 10^{-4} (M) for Ca-ATP and 5×10^{-5} (M) for Mg-ATP [14]. Supposing that tubulin dimer can bind 1 mole calcium per mole of tubulin dimer at maximum, the dissociation constant was estimated as 1.5×10^{-5} (M). This value approximately corresponds to the free calcium concentration which brings disassembly of reassembled tubulin as measured by flow birefringence [5].

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