Calcium binding to the tubulin-colchicine complex in the state of GTP in a BES buffer

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Magnesium and calcium binding were assayed using the tubulin-colchicine complex in a BES buffer, in which the calcium binding to tubulin had been measured by Grisham *et al.* (Fed. Proc. 39, 2162). In the previous paper, an imidazole buffer was used as the buffer which does not chelate to calcium and which is substituted to phosphate buffer. The result of calcium binding measurement indicated the same binding constant between at pH 7.0 and at pH 6.5 in the absence of magnesium(1.08×10^{-5} M at pH 7.0 and 1.10×10^{-5} M at pH 6.5). Also, the calcium binding constant of the tubulin-colchicine complex was the same as that of tubulin in a BES buffer, pH 7.0. The increase of magnesium concentration inhibited calcium binding to the tubulin-colchicine complex. The affinity of calcium at pH 7.0 was lower than that at pH 6.5 in the presence of magnesium. This leads the effect of calcium on the polymerization of the tubulin-colchicine complex is small at pH 6.5 rather than at pH 7.0. The results obtained in the polymerization experiment were in good agreement with the results of the calcium binding experiment.

Abbreviations: BES, N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Pipes, piperazine-N, N'-bis(2-ethanesulfonic acid); Mes, 2(N-morpholino)ethanesulfonic acid; GTP, guanosine 5'-triphosphate; TMX, tetramethylpurpurate.

Introduction

Grisham *et al.* have measured calcium binding to tubulin according to equilibrium gel filtration technique of Sephadex G-25(fine)using radioactive CaCl$_2$ under non-aggregating conditions of BES buffer[1]. Solomon[2] tried to measure calcium binding to tubulin in Tris buffer using equilibrium dialysis method before. Because tubulin is one of the unstable proteins, measurement should be carried out in a short time under the conditions that do not make protein denature.

One high affinity site with Kd=3.2×10^{-6} M and 16 low affinity sites were reported by Solomon, while Grisham *et al.* detected two high affinity sites with Kd=1.2×10^{-5} M and 18-20 low affinity sites. Serrano *et al.* have reported two high affinity sites located in carboxyl terminal region of each subunit of α and β[3].

It is reported that TMX is suitable to measure calcium binding for biological experiments[4]. These facts make us expect to get the intrinsic or very similar result of calcium binding to native tubulin from the calcium binding assay to the

tubulin-colchicine complex by using optical method with TMX.

In this paper, we will deal with the calcium binding to the tubulin-colchicine complex in a BES buffer.

Materials and Methods

Chemicals

BES buffer and GTP were purchased from Sigma. TMX was obtained from Calbiochem (Cat. No. 584401, Lot No. 309840). The preparations of calcium ion solution and purified water are carried out as described in our previous paper[9].

Preparation of the Tubulin-Colchicine Complex

Tubulin was prepared from calf brains that were obtained from freshly slaughtered animals by using the combined method described before[5-7]. The complex formation between tubulin and colchicine was performed according to the procedure of Andreu and Timasheff[8]. Removal of sucrose and excess colchicines and confirmation of the complex were performed as described in our previous paper[9].

Calcium Binding Assay

Among many methods for calcium binding assay, optical method using TMX as a metallochromic indicator was chosen[4], considering from the sensitivity, stability and reliability of the indicator and the necessary time to measure. It was considered from the result of Sephadex G-25 (fine) gel chromatography that TMX had no interaction with tubulin and the tubulin-colchicine complex under the condition used. Differential absorbance was determined with a Perkin Elmer Lambda 3B UV/VIS spectrophotometer equipped with a Perkin Elmer Digital Controller, Model C570-0701. Measurement was carried out repeatedly at 20℃. Protein concentration was between 20 and 40 μM. Scatchard[11] and Hill[12] plots were used to analyze the results of calcium binding assay. Calibration and measurement were carried out alternately. Reduction of sensitivity of TMX due to magnesium ion was detected, although the report of Ogawa et al. refers to no interference by magnesium.

Analytical Ultracentrifugation

In order to make sure α β heterodimer state of the tubulin-colchicine complex under the conditions of calcium binding assay, analytical ultracentrifugation experiment was performed with a Beckman Model E ultracentrifuge equipped with electronic speed control and RTIC. Protein concentration was between 1 and 5 mg/ml. Protein solutions were centrifuged in double sector cell with sapphire window at 20℃. Schlieren pattern was taken on a Kodak metallographic plate and analyzed with a Nikon Model 6C microcomparator equipped with Mitutoyo digimatic micrometer 164-152.

Results and Discussion

Calcium binding experiments were carried out using a metallochromic indicator in different concentrations of magnesium at pH 7.0 and 6.5. Figure 1A shows the result of the experiment at pH 7.0 without magnesium. Scatchard plot did not give a straight line in the region of high affinity. Then Hill plot is shown in Figure 1B. Hill plot is effective when it is difficult to determine the binding properties from Scatchard plot as well as the cooperative interaction between ligand and protein. Scatchard plot suggests at least two classes of affinity site in the tubulin-colchicine complex as shown by Solomon[2] in the tubulin molecule. This means that it is possible to make a mistake to determine the binding properties from Scatchard plot in this case. Figure 1B is drawn on the assumption with one, two, or three binding sites. When the binding number is assumed as one, plot does not give a straight line. It seems that the assumption as two or three may make plots straight. Increase of magnesium concentration led an affinity of calcium low (Figure 2A). A similar assumption to Figure 1B is taken in Figure 2B. Many points in Figure 2B made a straight line comparing to Figure 1B. It seems that the assumption or binding number as two is reasonable. Further increase of magnesium concentration resulted in the decrease of calcium affinity to the tubulin-colchicine complex (Figures
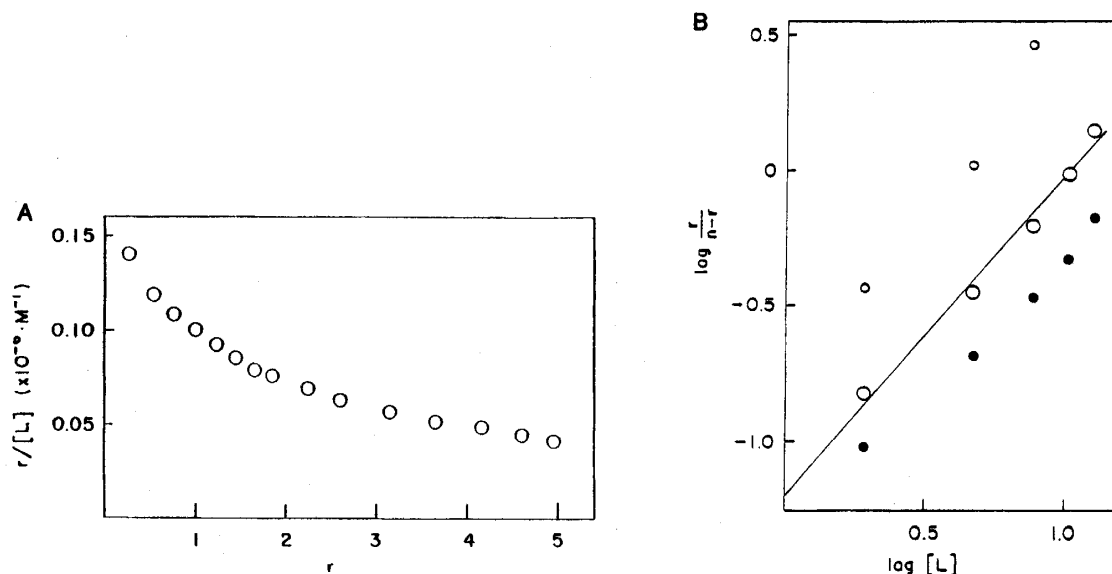
Fig. 1. Calcium Binding Assay to the Tubulin-Colchicine Complex in 10 mM BES Buffer, pH 7.0, in the Absence of Magnesium at 20℃ at Protein concentration of 30 μ M.

    A：Scatchard plot for calcium ion to the tubulin-colchicine complex. r：[moles calsium ion bound]/ [mole tubulin]；[L]：free calsium ion concentration(10⁻⁶ M).

    B：Hill plot for calcium ion to the tubulin-colchicine complex. ○, n＝1；○—○, n＝2；●, n＝3.
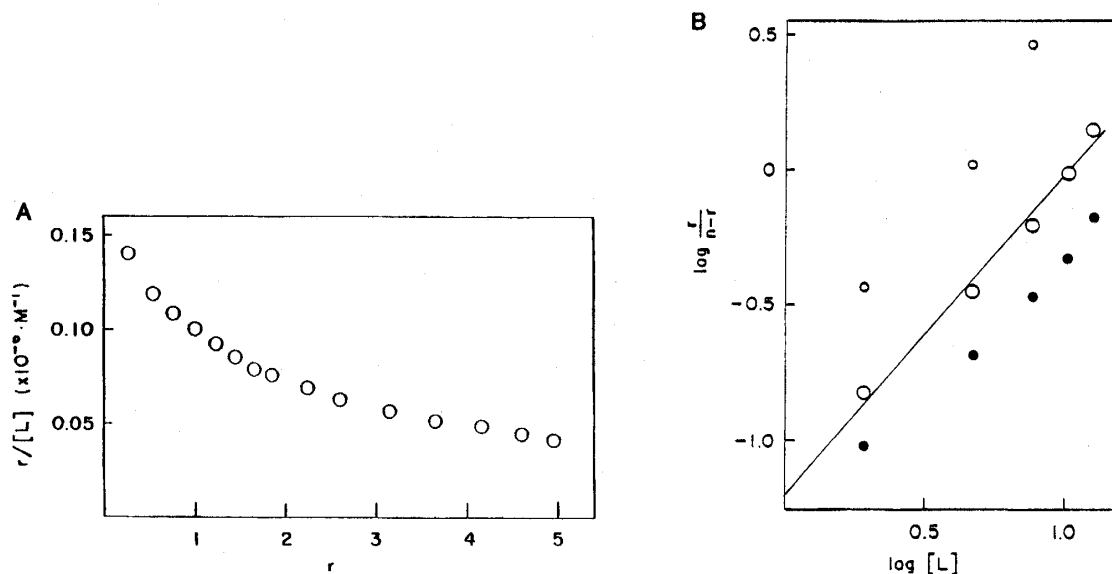

Fig. 2. Calcium Binding Assay to the Tubulin-Colchicine Complex in 10 mM BES Buffer, pH 7.0, in the Presence of 2 mM Magnesium and 0.1 mM GTP at 20℃ at Protein Concentration of 30 μ M.

    A：Scatchard plot for calcium ion to the tubulin-colchicine complex. r：[moles calsium ion bound]/ [mole tubulin]；[L]：free calsium ion concentration(10⁻⁶ M).

    B：Hill plot for calcium ion to the tubulin-colchicine complex. ○, n＝1；○—○, n＝2；●, n＝3.

3 and 4). We tried to determine the calcium binding at pH 6.5, too. The situation at pH 6.5 very closely resembled to one at pH 7.0. Figure 5 shows the result of calcium binding to the tubulin-colchicine complex at pH 6.5 without magnesium. Hill plot is drawn on the assumption with the binding number as two. The result at pH 6.5 in the presence of 2 mM MgCl₂ is shown
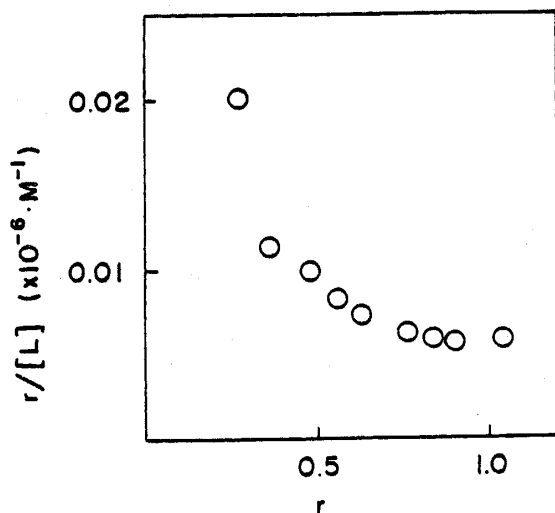
Fig. 3. Scatchard Plot for Calcium Ion to the Tubulin–Colchicine Complex in 10 mM BES Buffer, pH 7.0, in the Presence of 4 mM MgCl₂ and 0.1 mM GTP at 20℃ at Protein Concentration of 25 μM.
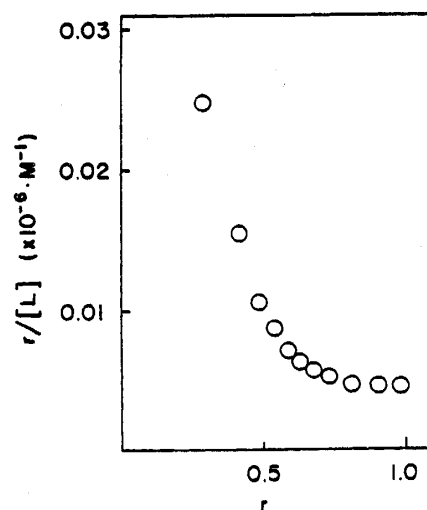
Fig. 4. Scatchard Plot for Calcium Ion to the Tubulin–Colchicine Complex in 10 mM BES Buffer, pH 7.0, in the Presence of 8 mM MgCl₂ and 0.1 mM GTP at 20℃ at Protein Concentration of 30 μM.
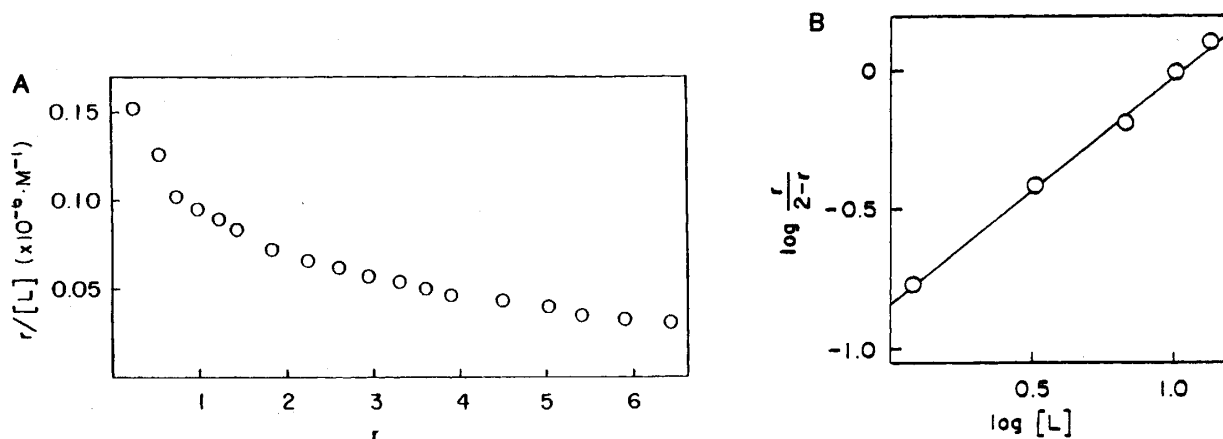

Fig. 5. Calcium Binding Assay to the Tubulin–Colchicine Complex in 10 mM BES Buffer, pH 6.5, in the Absence of Magnesium at 20℃ at Protein concentration of 30 μM.

A : Scatchard plot for calcium ion to the tubulin–colchicine complex. r : [moles calsium ion bound]/[mole tubulin] ; [L] : free calsium ion concentration (10^{-6} M).

B : Hill plot for calcium ion to the tubulin–colchicine complex.

in Figure 6. After getting a straight line, a plot raised up in Hill plot. It is considered that those points are contributed by low affinity sites. Considering from these results in Hill plot, we would like to conclude the high affinity binding sites as two. The binding constants are summarized in Table I. The number of two has been reported as a number of high affinities binding site of the

tubulin by Serrano et al.[3]. In the absence of magnesium, the binding constant at pH 7.0 was almost the same as that at pH 6.5. Also, these values are the same as the binding constant obtained under the condition of 10mM imidazole buffer, pH 7.0, at 20℃ in the absence of magnesium. The fact was different from the result of Solomon[2]. In this paper, it is considered that
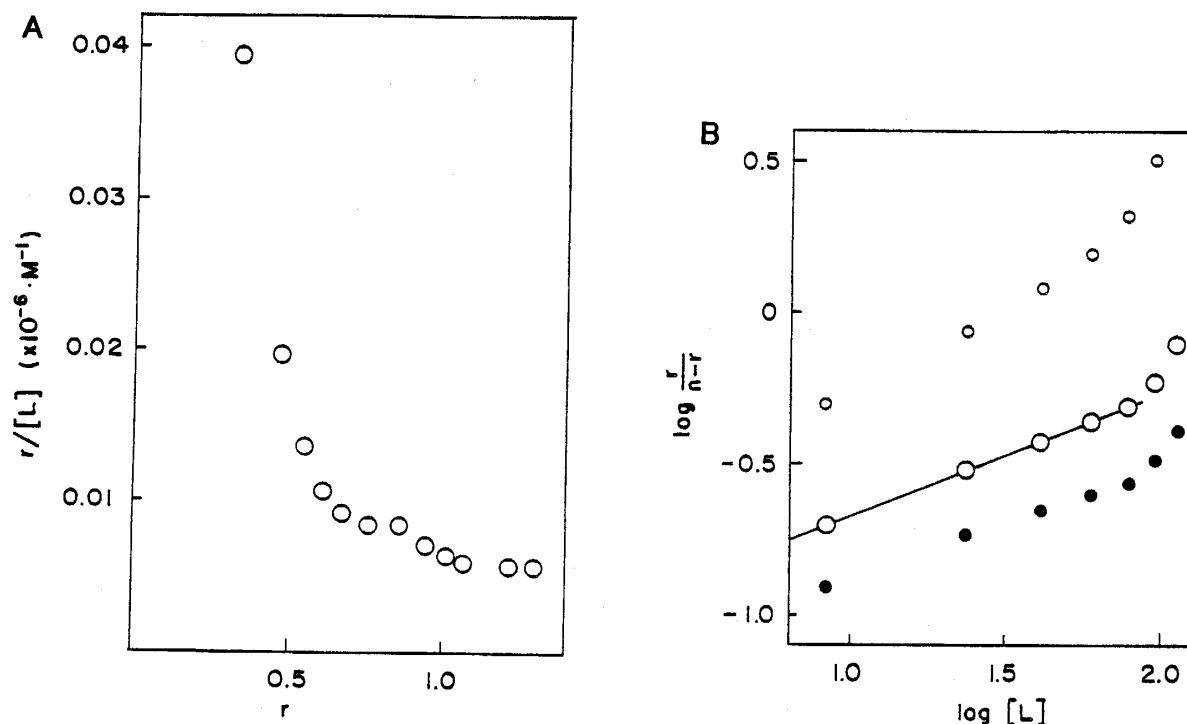
Fig. 6. Calcium Binding Assay to the Tubulin–Colchicine Complex in 10 mM BES Buffer, pH 6.5, in the Presence of 2 mM MgCl₂ and 0.1 mM GTP at 20℃ at Protein concentration of 35 μ M.

A∶Scatchard plot for calcium ion to the tubulin–colchicine complex. r∶[moles calsium ion bound]/ [mole tubulin]∶[L]∶free calsium ion concentration $(10^{-6}$ M).

B∶Hill plot for calcium ion to the tubulin–colchicine complex. ○, n=1;○—○, n=2;●, n=3.

tubulin–colchicine complex should be used to measure calcium binding instead of native tubulin, because the tubulin–colchicine complex is much more stable than native tubulin and it is assumed that the binding constant of calcium is same as order. In the case of Solomon, he tried to measure calcium binding to tubulin using equilibrium dialysis method. After 16 hours of dialysis, the solutions were counted to determine the distribution of calcium. The temperature used was 22 ℃. It is reasonable to consider that the experimental condition indicated by Solomon induced the denaturation of tubulin.

Increase of magnesium concentration led low affinity of calcium binding to the tubulin–colchicine complex in any case at pH 7.0 and 6.5. It seems that calcium binding competes with the binding of magnesium. In the case of imidazole buffer, there is no difference of the binding affinity of calcium between at pH 7.0 and pH 6.5. On the other hand, the tendency of low affinity

by magnesium is bigger at pH 6.5 than at pH 7.0. This means that the effect of calcium on the tubulin–colchicine complex is smaller at pH 6.5 than that at pH 7.0. For example, polymerization at pH 7.0 was much affected by calcium

Table 1. Binding Constants of Calcium to Tubulin–Colchicine Complex in BES Buffer in the state of GTP.

pH	MgCl₂ concn. (mM)	Binding Number	Dissociation Constant $(\times 10^{-6}$ M)
	0	2	10.8
		14	234
7.0	2	2	157
	4	2	335
	8	2	560
	0	2	11.0
6.5		13	237
	2	2	228
	4	2	720

MgCl$_2$
concn.
(mM)
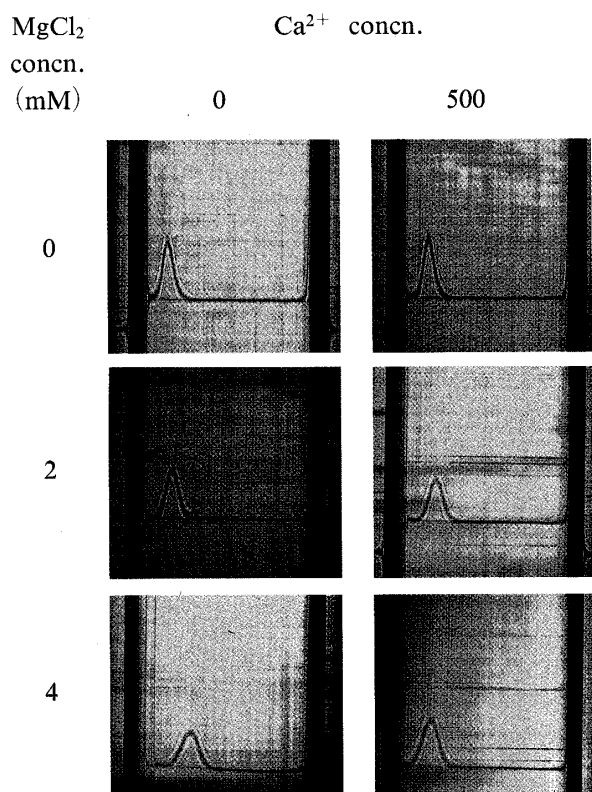
Ca^{2+} concn.

0   500

Fig. 7. Sedimentation Profiles of the Tubulin-Colchicine Complex in 10 mM BES-0.1 mM GTP Buffer, pH 7.0, in the Absence and Presence of Magnesium and Calcium at 20℃ at Protein Concentration of 4 mg/ml. Sedimentation was from left to right pictures were taken at 33±2 min after reaching the maximum speed at 52,000 rpm.

rather than that at pH 6.5[12]. In the presence of magnesium the binding affinity of calcium to the tubulin-colchicine complex was bigger than that in BES buffer. One possibility is that the binding affinity of magnesium to the tubulin-colchicine complex is bigger in BES buffer than that in imidazole buffer. Another possibility is that the conformation of the protein is due to the buffer and that the conformational change is led nearby the binding site of calcium by the buffer. Of course, it is possible that both possibilities occur in this case.

In order to make sure the protein conformation composed of α and β subunits and colchicine, analytical ultracentrifugation and sodium dodecylsulfate-polyamide gel electrophoresis at

pH 9.2 using 10% gel were performed. The composition of α and β subunits were confirmed by the electrophoresis. Complex formation was recognized from the absorption spectrum of the protein. The result of analytical ultracentrifugation is shown in Figure 7. In any case, sedimentation patterns gave a single peak with a sedimentation coefficient observed between 5.4 and 6.1 s. These results indicate that the protein was the state $\alpha\beta$ dimer of tubulin containing colchicine. The details of ultracentrifugation will be discussed later in another opportunity.

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