Effect of calcium on the polymerization of the tubulin–colchicine complex in a BES buffer

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The most fundamental property of tubulin is microtubule assembly, and then the polymerization reaction is the basic in the case of the tubulin–colchicine complex. Comparing the results carried out under different conditions is important, because microtubule formation is affected by the condition. The polymerization was observed above at a concentration of 4 and 6 mM MgCl₂ at pH 6.5 and 7.0, respectively, in a BES buffer. The increase of magnesium concentration induced the high turbidity due to the polymerization. We indicated calcium ion inhibited the polymerization at low concentration. The presence of calcium ion induced the increase of critical concentration for polymerization. We also observed, however, some tendency of acceleration of the polymerization due to a high concentration of calcium ion even in the presence of any concentration of magnesium ion. The ratio of inhibition by calcium was much more dependent on the magnesium concentration, especially the change of the inhibitory ratio was big at pH 7.0.

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

It is well known that critical concentration for microtubule formation from pure tubulin depends on buffers\(^1\). This means that the behavior of tubulin is due to the buffer conditions. Although there is no obvious systematic difference between the nature of ions, it appears that sulfonate buffers promote microtubule formation even if the concentration of buffer is low\(^1\). There is a report of Waxman et al.\(^2\) that sulfonate buffers such as Pipes and Mes induced the polymerization reaction of purified tubulin at high concentration and that the polymerization with sulfonate was more vigorous than those occurring with other buffers.

When it was discovered for calcium ion to inhibit microtubule assembly, sulfonate buffers were used\(^3\). Among many divalent cations that induce microtubule assembly\(^4\)\(^–\)\(^9\), it is considered that magnesium ion plays some roles on \emph{in vivo} microtubule assembly, because magnesium is the major intracellular divalent cation\(^10\). Also, it is possible that calcium ion regulates the microtubule assembly by competing with magnesium ion. Therefore, it is important to make clear the effect of calcium ion on microtubule assembly in the presence of magnesium.

Tubulin can form microtubule without microtubule–associated proteins in the presence of glycerol, magnesium, and GTP\(^11\); and the microtubule formation is inhibited by calcium
ion\(^3\). This means calcium is able to bind the tubulin molecule directly. Then it is interesting to measure calcium binding to tubulin.

The stability of tubulin is improved by the complex formation with colchicine\(^{12}\). The tubulin-colchicine complex has very similar properties to those of tubulin\(^{13-17}\).

Based on the functions of microtubules, it is important to examine the buffer condition for microtubule formation. In this paper, we will deal with the effect of calcium on the polymerization of the tubulin-colchicine complex instead of microtubule formation in a BES buffer.

Materials and Methods

Chemicals

BES buffer and GTP were purchased from Sigma. Calcium carbonate and calcium chloride were from Fisher Scientific Co. Calcium ion solution was prepared from dried calcium carbonate by adding the minimum necessary amount of HCl. Purified water was obtained by further distillation using glass distillation apparatus, after distilled water was purified by the system of charcoal and ion exchange resin columns of Hydro Service and Supplies Inc. Throughout all experiments including preparation of tubulin, this repeated purified water was used.

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\(^{18-20}\). The complex formation between tubulin and colchicine was performed according to the procedure of Andreu and Timasheff\(^{12}\). Removal of sucrose and excess colchicine and equilibration from phosphate buffer to BES buffer were carried out by twice gel chromatographies of dry and wet Sephadex G-25 (fine)\(^{21}\). Complex formation was confirmed spectrophotometrically comparing with the result of Andreu and Timasheff\(^{12}\). Also protein concentration was determined spectrophotometrically using a Perkin Elmer Lambda 3B UV/VIS spectrophotometer\(^{12}\).

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**Fig. 1.** Time Course of Polymerization of the Tubulin-Colchicine Complex in 10 mM BES-0.1 mM GTP Buffer, pH 7.0, in the presence of Various Concentrations of Magnesium. Protein concentrations was 1.8 mg/ml. Arrow ⇒ indicates the addition of 100 μM calcium ion and arrow → does the cooling of sample to 10°C. ---, 16 mM MgCl\(_2\); ---, 12 mM MgCl\(_2\)-100 μM Ca\(^2+\); ---, 8 mM MgCl\(_2\).

**Fig. 2.** Time Course of Polymerization of the Tubulin-Colchicine Complex in 10 mM BES-0.1 mM GTP Buffer, pH 6.5, in the Presence of Various Concentrations of Magnesium. Protein concentrations was 1.8 mg/ml. Arrow ⇒ indicates the addition of 100 μM calcium ion and arrow → does the cooling of sample to 10°C. ---, 8 mM MgCl\(_2\); ---, 8 mM MgCl\(_2\)-100 μM Ca\(^2+\); ---, 4 mM MgCl\(_2\)-100 μM Ca\(^2+\).
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![Graph](image)

**Fig. 3.** Effect of Magnesium Concentration on Polymerization of the Tubulin-Colchicine Complex in 10 mM BES-0.1 mM GTP Buffer, pH 7.0 and 6.5. Protein concentration was 1.8 mg/ml. ○, pH 7.0; ●, pH 6.5.

**Polymerization of the Tubulin-Colchicine Complex**

Polymerization of the tubulin-colchicine complex was carried in 10 mM BES-0.1 mM GTP buffer, pH 7.0 or 6.5, not containing glycerol in the presence of magnesium ion by warming the protein solution in a jacketed cuvette from 10 to 37 °C.

**Results and Discussion**

**The polymerization of the Tubulin-Colchicine Complex in BES Buffer**

The polymerization of the tubulin-colchicine complex has been reported\(^{[16, 17]}\). GTP and magnesium were required for the polymerization. Although microtubule assembly from pure native tubulin required glycerol, the polymerization of the tubulin-colchicine complex was observed in the absence of glycerol. We made clear the polymerization of the tubulin-colchicine complex in BES buffer at pH 7.0 and 6.5 without glycerol in the absence and presence of calcium ion before calcium binding assay was investigated in the absence and presence of magnesium. 10\(^{-5}\) M GTP was not enough to make the tubulin-colchicine complex to polymerize\(^{[17]}\). We observed that polymerization at the concentration of 10\(^{-3}\) M GTP was smaller in the presence of 4 mM MgCl\(_2\) at pH 6.5 than that at one of 10\(^{-4}\) M GTP (data not shown). However, the existence of 8 mM MgCl\(_2\) gave the same turbidity due to the polymerization in the presence of between 0.1 and 1.0 mM GTP. This does not mean that GTP inhibits the polymerization. It is reasonable that magnesium concentration necessary for the polymerization was reduced by the GTP binding to magnesium ion\(^{[22]}\). Then, in our polymerization experiments, the concentration of GTP was 0.1 mM throughout. This concentration was suggested by the results of Andreu and Timasheff\(^{[17]}\), too.

Figures 1 and 2 show the typical time course of the polymerization from the tubulin-colchicine complex at pH 7.0 and 6.5 respectively. As shown in these figures, the tubulin-colchicine complex was able to polymerize in 10 mM BES buffer in the presence of magnesium and GTP without glycerol at pH 7.0 and 6.5. The polymerization was reversible by cooling and inhibited by the addition of calcium ion (Figure 1). The same turbidity was obtained by the addition of calcium between before and after warming (Figure 2). Not to say, it seems that the extent of poly-
Fig. 5. Comparison of Polymerization under the Conditions between with and without Calcium in 10 mM BES-0.1 mM GTP Buffer, pH 7.0, in the Presence of Various Concentrations of Magnesium. Protein concentration was 1.8 mg/ml. ○, no calcium; ●, 100 μM calcium ion.

Fig. 6. Comparison of Polymerization under the Conditions between with and without Calcium in 10 mM BES-0.1 mM GTP Buffer, pH 6.5, in the Presence of Various Concentrations of Magnesium. Protein concentration was 1.8 mg/ml. ○, no calcium; ●, 100 μM calcium ion.

Polymerization is affected by magnesium concentration. The effect of magnesium ion concentration on the polymerization was shown in Figure 3. The polymerization was much dependent on magnesium ion concentration as well as pH value. No polymerization was observed at the concentration of 2 and 4 mM MgCl₂ at pH 6.5 and 7.0, respectively. The increase of magnesium concentration induced high turbidity due to the polymerization of the tubulin–colchicine complex at both pH 7.0 and pH 6.5. As shown previously, the fact that higher pH needed higher magnesium concentration for the polymerization was obtained in 10 mM imidazole buffer, pH 6.5 and 7.0, containing 0.1 mM GTP as well as in BES buffer. It is shown by Gaskin et al. that microtubule formation at pH 6.5 is greater than that at pH 7.0. A similar tendency was obtained in the case of the polymerization of the tubulin–colchicine complex. The results obtained in this paper are in good agreement with those.

In the microtubule assembly experiment, turbidimetry is due to total mass of high molecular weight material and viscosity development is influenced on both increased number and length of microtubules. As microtubule formation is characterized by the critical concentration, the comparison of turbidity is reasonable. The turbidity obtained with BES buffer was slightly lower compared with that obtained in imidazole buffer. For example, the increase of turbidity due to polymerization was 0.96 in 10 mM BES-0.1 mM GTP-8 mM MgCl₂ buffer, pH 6.5, at the protein concentration of 1.00 mg/ml, while the value with imidazole buffer was 1.57. At pH 7.0, the increase of turbidity were 1.330 and 1.501 in 10 mM BES and imidazole buffers, containing 0.1 mM GTP and 8 mM MgCl₂ at the protein concentration of 1.50 mg/ml, respectively.

Critical concentration was determined at pH 7.0 at 8 mM MgCl₂ as shown in Figure 4. Table 1. indicates the critical concentration for the tubulin–colchicine complex in 10 mM BES-0.1 mM GTP-8 mM MgCl₂ buffer, pH 6.5 and 7.0, in the absence and presence of 100 μM calcium.
Table 1. Critical Concentration for Polymerization of the Tubulin-Colchicine Complex in 10 mM BES Buffer Containing 0.1 mM GTP.

<table>
<thead>
<tr>
<th>pH</th>
<th>MgCl₂ concn. (mM)</th>
<th>Ca²⁺ concn. (M)</th>
<th>critical concentration (mg/ml)</th>
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</thead>
<tbody>
<tr>
<td>7.0</td>
<td>8</td>
<td>0</td>
<td>0.70</td>
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<tr>
<td></td>
<td></td>
<td>100</td>
<td>1.03</td>
</tr>
<tr>
<td>6.5</td>
<td>8</td>
<td>0</td>
<td>0.27</td>
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<td></td>
<td></td>
<td>100</td>
<td>0.35</td>
</tr>
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ion. As expected, pH 6.5 (0.27 mg/ml) gave lower critical concentration than pH 7.0 (0.70 mg/ml). Also, the critical concentration at pH 7.0 was a little larger in BES buffer than in imidazole buffer (0.608 mg/ml) at the same concentration of magnesium. In the presence of 100 μM calcium ion, the increase of turbidity were 0.87 and 1.394 in 10 mM BES and imidazole buffers, pH 6.5, containing 0.1 mM GTP and 8 mM MgCl₂ at a protein concentration of 1.0 mg/ml, respectively. By raising pH from 6.5 to 7.0, the value became small to 0.76 and 1.164 in 10 mM BES and imidazole buffers, pH 7.0, containing 0.1 mM GTP and 8 mM MgCl₂, respectively, even if the protein concentration increased to 1.50 mg/ml. This suggests that BES buffer gives a larger critical concentration than that imidazole buffer does at pH 6.5 and 7.0 in the presence of 100 μM calcium ion. This order of buffer that gives critical concentration is different from the one in the case of microtubule assembly.¹

The addition of 100 μM calcium ion led critical concentration to increase. Figures 5 and 6 show the increase of turbidity due to polymerization in the absence and presence of 100 μM calcium ion. Calcium ion indicated an inhibitory effect on the tubulin-colchicine complex polymerization at any concentration of magnesium, while the extent of inhibition was due to pH value and magnesium concentration. The inhibitory effect of 100 μM calcium ion on polymerization is shown in Figure 7. It is very clear that the inhibitory effect of calcium ion is much influenced by the concentration of magnesium and pH value. When pH value was 7.0, the existence of 100 μM calcium inhibited 65.2 and 24.7% of polymerization at the concentration of 6 and 8 mM MgCl₂, respectively. Reducing pH from 7.0 to 6.5 induced only 5.5% of inhibition at 8 mM MgCl₂. Even if magnesium concentration was 4 mM, only 14.9% of inhibition was observed. These results indicate that calcium binding to tubulin-colchicine complex is affected by pH and magnesium concentration. In fact, the results of calcium binding capacity of the tubulin-colchicine complex indicated that the capacity was much influenced by the magnesium concentration in the presence of glycerol at 37 and 20°C (data not shown), but that the effect of pH was not so much. These facts suggested the necessity of calcium binding experiment to the tubulin-colchicine complex.

References
2) Waxman, P.G., Del Camp, A.A., Lowe,