J. Biol. Macromol., **3**(3) 117-125 (2003)

Ultracentrifugal Behaviors of the Tubulin-Colchicine Complex in the State of GTP in Different Buffers

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Received August 20, 2003, Accepted September 16, 2003

The behavior of the tubulin-colchicine complex was investigated by using an analytical ultracentrifugation technique. The partial specific volumes of the tubulin-colchicine complex in the state of GTP were 0.739 and 0.744 ml/g in imidazole and BES buffers, respectively. The values $S^0_{20.w}$ were 5.82 and 6.06 S in an imidazole buffer in the presence of 0 and 2 mM $MgCl_2$, respectively. In the BES buffer, however, very interesting results were obtained. The sedimentation coefficient $S^0_{20.w}$ increased from 5.38 S with a calcium concentration of 0 μM to 5.75, 6.08 S with calcium concentration of 100 and 500 μM, respectively, in the absence of magnesium. This means that the protein molecule has a very flexible shape in the BES buffer and that the shape shrinks by the addition of calcium. The increase of either the calcium or the magnesium ion concentration induced the increase of the sedimentation coefficient. This result was in good agreement with the inducing effect of calcium on the polymerization of the tubulin-colchicine complex in the state of GDP. The sedimentation coefficient of the GDP state was higher than that of the GTP state.

Many studies on the conformation of tubulin have been done by using an analytical ultracentrifugation technique[1-4]. Tubulin exists as a 5.8 S dimer or a 42 S double ring depending on the magnesium concentration in solution[2, 3, 5]. Some researchers have found 18, 30, and 36 S oligomers during ultracentrifugation experiments of tubulin in the presence of microtubule-associated proteins[6-8]; on the other hand, results of ultrafiltration experiments and others have recently raised the question of the state of oligomers in solution at room temperature [9]. Many properties of tubulin, for example, microtubule assembly, sedimentation coefficient, and bindings of nucleotide and calcium are easily affected by the magnesium ion concentration in solution[2, 10, 11]. Currently the effect of calcium on tubulin have been reported [12,13]. Also we have indicated the

† To whom correspondence should be addressed. TEL and FAX: +81-798-45-9883; e-mail: hiroshid@mwu.mukogawa-u.ac.jp Abbreviations: GTP, guanosine 5'-triphosphate; GDP, guanosine 5'-diphosphate; TC-GTP, tubulin-colchicine complex in the state of GTP; TC-GDP, tubulin-colchicine complex in the state of GDP; Tu-GTP, tubulin in the state of GTP; Tu-GDP, tubulin in the state of GDP.

calcium binding to the tubulin-colchicine complex in the state of GDP [14] and the effect of calcium ion on the complex in the GTP state in the BES buffer [15,16]. During a calcium binding study of the tubulin-colchicine complex, we found that the behavior of the protein was interesting in the experiment of analytical ultracentrifugation.

In this paper, the behaviors of the tubulin-colchicine complex in the state of GTP in different buffers are described. Also, the similarity of the tublin-colchicine complex to tubulin is indicated on the basis of an analytical ultracentrifugation experiment.

Materials and Methods

Preparation of Calf Brain Tubulin. Tubulin was prepared from the brain of freshly slaughtered calves according to the modified procedure described previously[1, 3, 5]. The tubulin preparation was stored in a 10 mM phosphate buffer, pH 7.0, containing 0.1 mM GTP, 0.5 mM $MgCl_2$, and 1 M sucrose under liquid nitrogen until used. Protein concentration was determined spectrophotometrically as described by Andreu and Timasheff[13]. Equilibrium to the desired buffer was carried out using a batch and a column of Sephadex G-25 (fine) gel equilibrated with the desired buffer.

Preparation of the Tubulin-Colchicine Complex. The complex formation between tubulin and colchicine was performed by the incubation of tubulin (40-53 mg/ml) and 1 mM colchicine in a 10 mM phosphate buffer, pH 7.0, containing 0.1 mM GTP, 0.5 mM $MgCl_2$, and 1 M sucrose at 25 ℃ for 10 min as described by Andreu and Timasheff [17]. Equilibrium to the desired buffer was accomplished as described above. To prepare the GTP state, 0.1 mM GTP was included in every step of preparation.

Density Measurement. To determine the partial specific volume of protein, the density of the protein solution was measured at various concentrations using an Anton Paar Precision Density Meter DMA 02C. All measurements were carried out at 20 ℃. The details of the measurements are described in Lee and Timasheff [18].

Analytical Ultracentrifugation. Analytical ultracentrifugation was performed with a Beckman Model E ultracentrifuge equipped with electronic speed control and RTIC. Protein solutions were centrifuged in a double sector cell with a sapphire window at 20 ℃. Schlieren patterns were traced on a Kodak metallographic plate. The radial positions of Schlieren patterns were recorded as the peak position. Analysis of the sedimentation velocity was performed with a Nikon Model 6C microcomparator equipped with a Mitutoyo Digimatic Micrometer 164-152. The measured sedimentation velocities were corrected to water at 20 ℃ using the values of the partial specific volume obtained in this study. The following equation was used to correct the sedimentation velocity, S_{obs}, to the sedimentation coefficient ($S^0_{20.w}$) of protein in water at 20 ℃[19]:

$$S^0_{20.w} = S_{obs} \cdot \eta / \eta_{20.w} \cdot (1 - v\rho)_{20.w} / (1 - v\rho),$$

where η is the viscosity of the buffer used, $\eta_{20.w}$ is the viscosity of water at 20 ℃, v is the partial specific volume of the protein, and ρ is the density. Subscripts 20 and w indicate 20 ℃ and water, respectively.

The viscosity of the buffers was measured using an Ostwald viscometer at 20 ℃.

Results

Determination of Partial Specific Volume

The partial specific volume is recognized as an important parameter of the protein-solvent interaction and is essential for the determination of the sedimentation coefficient. Though the partial specific volume of tubulin was determined by Lee and Timasheff [18], it is possible that the value changes due to the buffer because the partial specific volume of protein is dependent on the solvent conditions. Basically, the values of tubulin and the tubulin-colchicine complex can be very similar. However, we have no exact data of the partial specific volume of the tubulin-colchicine complex.

Fig. 1 shows the relationship between the apparent partial specific volume and protein concentrations of the tublin-colchicine complex in the state of GTP in a 10 mM imidazole buffer, pH 7.0, containing 0.1 mM GTP at 20 ℃. Analysis was performed by a least squares analysis because an extrapolation is necessary to obtain the true value of a partial specific volume[14]. The extrapolation of the plots gives the value of 0.739 ml/g. This extrapolated value was the same as the mean value of apparent partial specific volumes. The partial specific volume of the tubulin-colchicine complex in the state of GDP in a BES buffer was 0.744 ml/g (Fig. 2). The values obtained here are very close in both buffers.

Analytical Ultracentrifugation of the Tubulin-Colchicine Complex

There are many reports on the sedimentation of tubulin[1,2,5,7,8,24]. Previous studies using ultracentrifugation indicate that tubulin consists of an α-β dimer with 5.8 S. Also, it is indicated that colchicine binding to tubulin has no apparent effects on the sedimentation of tubulin[1, 9, 24]. It is well known that the behavior of tubulin is dependent on the buffer[10,21,22]. In this paper,

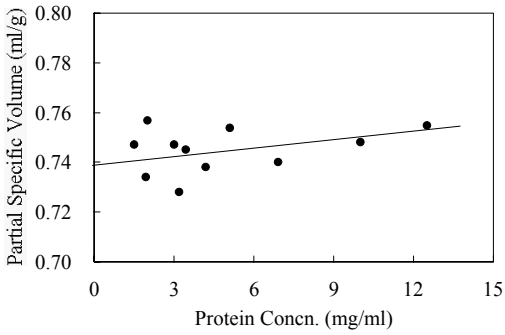

Fig. 1. Relationship between apparent partial specific volume and protein concentration of the tubulin-colchicine complex in the state of GTP in 10 mM imidazole buffer, pH7.0, containing 0.1 mM GTP at 20 ℃.

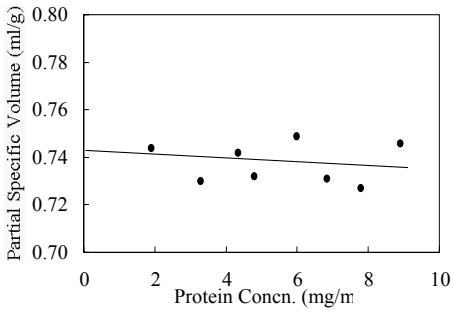

Fig. 2. Relationship between apparent partial specific volume and protein concentration of the tubulin-colchicine complex in the state of GTP in 10 mM BES buffer, pH7.0, containing 0.1 mM GTP at 20 ℃.

the buffer effect and the difference between GTP and GDP states are examined by sedimentation analysis. Schlieren peaks are single and symmetrical in the absence and presence of magnesium and/or calcium. Fig. 3 shows the concentration dependence of the sedimentation velocity of TC-GTP at various concentrations of magnesium and calcium in a 10 mM imidazole buffer, pH 7.0, containing 0.1 mM GTP at 20 ℃. In the absence of magnesium, the concentration dependence was

negative. Usually, protein indicates the concentration dependence in an analytical ultracentrifugation experiment. The results obtained in this study indicate the typical profile of tubulin. The observed sedimentation coefficients were corrected to $S^0_{20.w}$. The corrected sedimentation coefficients were 5.82, 5.81, and 5.88 S in the presence of 0, 100, and 500 μM calcium ion, respectively. It seems that the addition of calcium induced a small increase in the sedimentation coefficient in the absence of magnesium. However, there was no difference in the sedimentation coefficient of TC-GTP in the presence of 2 mM $MgCl_2$. The value of $S^0_{20.w}$ of TC-GTP was 6.06 S in a 10 mM imidazole buffer, pH 7.0, containing 0.1 mM GTP and 2 mM $MgCl_2$. These results indicate that the addition of salt in solution introduced a small change in the shape of the molecule of TC-GTP in an imidazole buffer, although it seems that the effect of the addition of salt did not change the molecular weight because the change in the sedimentation coefficient was very small. A further addition of magnesium led to a positive concentration dependence of the sedimentation coefficient, and the dependence was not a straight line, as has been observed in many tubulin studies[2, 23,24]. Therefore, the sedimentation coefficient of $S^0_{20.w}$ can be determined as 6.06 S. Each pattern under different conditions was a single symmetrical peak, as observed in the imidazole buffer. However, the behavior of the protein in the BES buffer was quite different from that in the imidazole buffer. The difference in the absence of magnesium was particularly remarkable. The concentration dependence indicated a negative straight line under the condition without magnesium, as shown in Fig. 4. The values of $S^0_{20.w}$ of TC-GTP in a 10 mM BES buffer, pH 7.0, containing 0.1 mM GTP were 5.38, 5.75, and 6.08 in the presence of 0, 100, and 500 μM calcium ion, respectively. This means that the molecular shape of TC-GTP in the BES buffer is very flexible and is easily affected by the existence of calcium ion. In the presence of 2 mM $MgCl_2$, the sedimentation coefficient, $S^0_{20.w}$, of the protein was 6.20 S in the absence of calcium ion. The increase of salt concentration led to the increase of the sedimentation coefficient in the BES buffer, too. Moreover, Figs. 3 and 4 show the interesting result that the lines without calcium indicate a highest sedimentation coefficient in the presence of 4 mM $MgCl_2$ in both buffers, even if it appears that calcium induces the increase of sedimentation coefficient in the

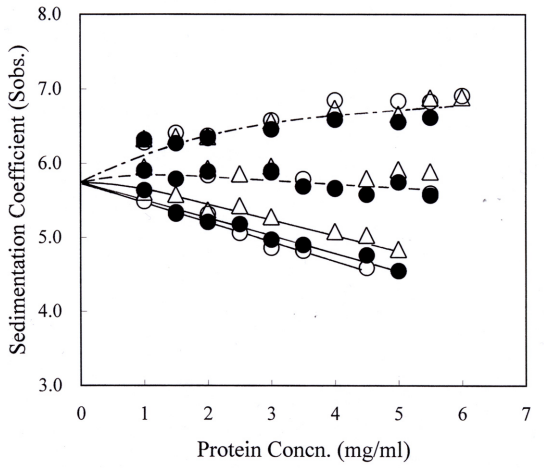

Fig. 3. Concentration dependence of the sedimentation velocity of the tubulin-colchicine complex in the state of GTP at various concentrations of magnesium and calcium ions in 10 mM imidazole buffer, pH7.0, containing 0.1 mM GTP.
The centrifugation was performed at 52,000 rpm at 20 ℃. Sedimentation velocity is expressed as $s_{observed}$. ____ ; no $MgCl_2$, ;2mM $MgCl_2$, _ _ _ ; 4 mM $MgCl_2$, ○ ; no Ca^{2+}, ● ; 100 μM Ca^{2+}, △ ; 500 μM Ca^{2+}.

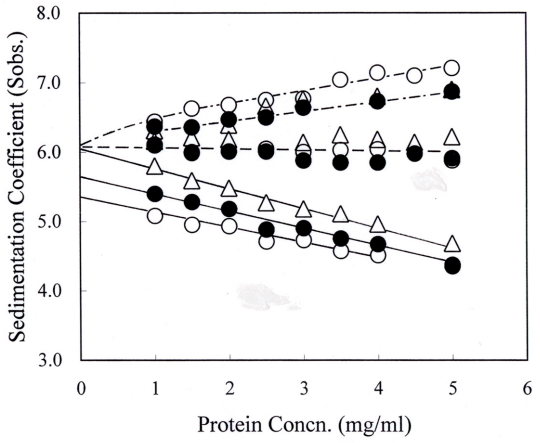

Fig. 4. Concentration dependence of the sedimentation velocity of the tubulin-colchicine complex in the state of GTP at various concentrations of magnesium and calcium ions in 10 mM BES buffer, pH7.0, containing 0.1 mM GTP.
The centrifugation was peroformed as described in Fig. 3.

absence of magnesium. These results suggest two roles of calcium ion in cases with and without magnesium.

It is interesting to compare the behavior of the tubulin-colchicine complex to that of tubulin in the buffer. The results of the analytical ultracentrifugation of tubulin in the imidazole buffer are shown in Fig. 5. The values of $S^0_{20.w}$ of Tu-GTP in a 10 mM imidazole buffer, pH 7.0, containing 0.1 mM GTP without magnesium were 5.84 and 5.99 S in the absence and presence of 500 μ M calcium ion, respectively. This indicates that the $S^0_{20.w}$ of Tu-GTP in the imidazole buffer is the same in the absence and presence of calcium ion, as observed in the case of TC-GTP in imidazole buffer.

On the other hand, in the case of the BES buffer, the result is shown in Fig. 6. Similarly to TC-GTP in the BES buffer, the behavior of Tu-GTP in the BES buffer is different from that in an imidazole buffer. The $S^0_{20.w}$ values of Tu-GTP in a 10 mM BES buffer, pH 7.0, containing 0.1 mM GTP without magnesium were 5.47 and 6.07 S in the presence of 0 and

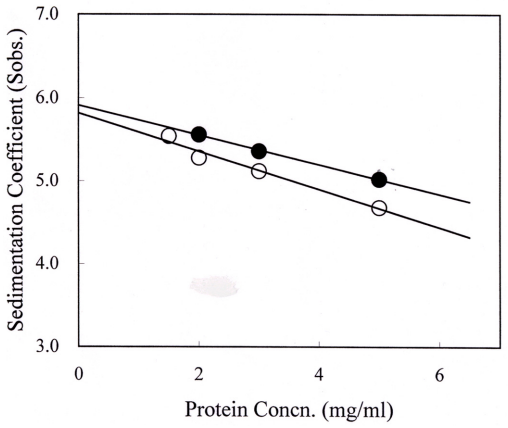

Fig. 5. Concentration dependence of the sedimentation velocity of tubulin in the state of GTP in 10 mM imidazole buffer, pH7.0, containing 0.1 mM GTP in the presence of 0 or 500 μ M Ca^{2+} without magnesim ion.
The centrifugation was performed at 52,000 rpm at 20 ℃. ○ ; no Ca^{2+}, ●; 500 μ M Ca^{2+},

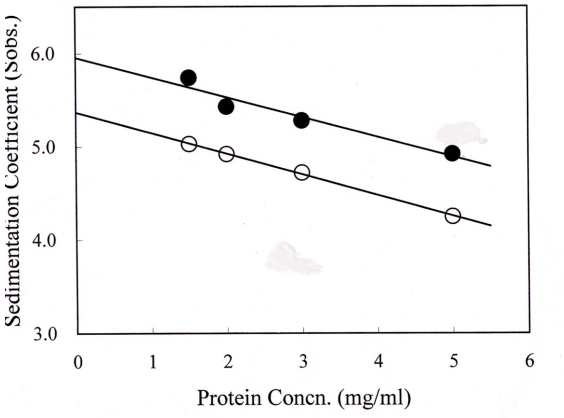

Fig. 6. Concentration dependence of the sedimentation velocity of the tubulin-colchicine complex in the state of GTP in 10 mM BES buffer, pH7.0, containing 0.1 mM GTP in the presence of 0 or 500 μ M Ca^{2+} without magnesium ion.

500 μ M calcium ion, respectively. These values are quite similar to those of TC-GTP

under the same conditions. The results shown in Figs. 5 and 6 suggest that the behavior of TC-GTP is very similar to that of Tu-GTP in both buffers.

Discussion

The behavior of the tubulin-colchicine complex in buffers was investigated using an analytical ultracentrifugation technique. It is well known that the tubulin molecule exists as a 5.8 S dimer in solution[2, 5, 7, 23]. The tubulin-colchicine complex indicates very similar properties to those of tubulin[20]. The study of tubulin is carried out using various buffers, and it seems that the behavior of tubulin is dependent on the buffer used[21,22]. We believe that it is best to use the phosphate buffer because tubulin is more stable in the phosphate buffer. However, because of the peculiarities of the experiment, we will not be able to use a phosphate buffer in the study of tubulin. In this study, imidazole and BES buffers were used, because calcium ion was contained.

Basically, it is believed that the behavior of tubulin in an imidazole buffer is similar to that in a phosphate buffer. On the other hand, there have been many studies that have used sulfonate buffers. As the behavior of tubulin is very complicated, it is interesting to compare the buffer effect on the behavior of tubulin.

The partial specific volume of tubulin has been determined as 0.736 ml/g by Lee et al.[3], while the value in lysine-HCl and glutamate buffers was 0.754 ml/g[21]. In the case of TC-GTP, the partial specific volumes were 0.739 and 0.744 ml/g in 10 mM imidazole and BES buffers, pH 7.0, containing 0.1 mM GTP, respectively. Though it appears that there is a small difference in the partial specific volume of TC-GTP between both buffers, we would like to consider that there is no difference because the value of the standard error was 0.0031 ml/g. It is clear that the partial specific volume obtained here is between two values of tubulin reported before, even if the difference between them is large. Therefore, it is also indicated that the tubulin-colchicine complex is similar to tubulin from the point of view of the partial specific volume.

The ultracentrifugal behavior of TC-GTP was the same as that of Tu-GTP in the imidazole buffer. As expected, the behavior of TC-GTP in the imidazole buffer is ultracentrifugally the same as that in the phosphate buffer when the data of reference[20] and our preliminary results of ultracentrifugation in a phosphate buffer are compared. A thermodynamic study has shown that the tubulin-colchicine complex has very similar properties to tubulin[26-28]. Tubulin still has GTPase activity after the complex formation with colchicine[29]. On the other hand, there are some differences of the geometric structure after polymerization[20,26,27]. The critical concentration for the polymerization of TC-GTP was smaller in the imidazole buffer than in the BES buffer(unpublished data), while the order of the critical concentration for the microtubule assembly of Tu-GTP was the reverse between the imidazole and sulfonate buffers[21]. Therefore, in conclusion, the tubulin-colchicine complex is comparable with tubulin in physicochemical properties, except for its apparent nature.

In the absence of magnesium, the concentration dependence of the sedimentation coefficient was observed in the imidazole and BES buffers. There was a big difference in the concentration dependence between the imidazole and BES buffers due to the influence of the calcium ion concentration. While the effect of the calcium ion concentration on the sedimentation coefficient of TC-GTP was small in the 10 mM imidazole buffer, pH 7.0, containing 0.1 mM GTP, the change in the

calcium ion concentration brought a significant difference in the sedimentation coefficient of TC-GTP in the 10 mM BES buffer, pH 7.0, containing 0.1 mM GTP. This tendency was observed in the case of Tu-GTP as well. In the case of the GTP state in the imidazole buffer, the concentration dependence was very similar between Tu-GTP and TC-GTP in the absence of magnesium. This suggests that the molecular shape of Tu-GTP was similar to that of TC-GTP. In the BES buffer, the behavior of Tu-GTP was similar to that of TC-GTP in the absence of magnesium. Therefore, it is considered that the behavior of the tubulin-colchicine complex in solution was similar to that of tubulin in the imidazole and BES buffers in the state of GTP. In other words, the sedimentation coefficients of TC-GTP and Tu-GTP were 5.38 and 5.47 S, respectively, in the BES buffer in the absence of magnesium and calcium. This means that the molecular shapes of TC-GTP and Tu-GTP are very flexible in the BES buffer without salt. As shown in Figures 3 and 4, the concentration dependence is smaller in the imidazole buffer than in the BES buffer. This means that the molecular shape of TC-GTP is easily affected by the salt concentration in the BES buffer. It is known that the critical concentration for microtubule formation of tubulin is lower in a sulfonate buffer than it is in a phosphate buffer[21]. The flexibility of the molecular shape may lead to a low critical concentration due to the easiness of self-association of the protein. In other words, it is believed that the difference in the behavior of TC-GTP in the buffers leads to the difference in microtubule formation. The association of the protein molecule is reflected in the value of the sedimentation coefficient. The larger the sedimentation coefficient, the larger the extent of the association. Though the polymerization of TC-GTP is not observed at 4 mM $MgCl_2$, it is believed that the association of TC-GTP is the prestate of polymerization. It is then reasonable to compare the sedimentation coefficient of the protein in the different buffers at 4 mM $MgCl_2$ without calcium ion; the values of S_{obs} of TC-GTP in the BES buffer were bigger than those in the imidazole buffer. This result is in good agreement with the difference in the critical concentration between both buffers. Furthermore, the presence of calcium ion reduced the value of S_{obs} of TC-GTP at 4 mM $MgCl_2$ (Figs. 3 and 4). These results are also in good agreement with the inhibition effect of the calcium ion on the polymerization of TC-GTP. In fact, the difference of S_{obs} between the absence and presence of 100 μ M calcium ion was larger in the BES buffer than in the imidazole buffer in the presence of 4 mM $MgCl_2$. The inhibition ratio of the calcium ion on polymerization was larger in the BES buffer than in the imidazole buffer in the presence of 6 mM $MgCl_2$ at 7.0. Moreover, the value of S_{obs} in the presence of 500 μ M calcium ion was between that in the presence of 0 and 100 μ M calcium ion.

The maximum inhibition of calcium has been observed at approximately 100 μ M calcium ion at 8 mM $MgCl_2$, though the presence of 500 μ M calcium ion still indicates a small inhibition effect[14]. The fact described above is demonstrated by the results of an ultracentrifugation experiment in the presence of 4 mM $MgCl_2$. It seems that calcium ion has an association effect in the absence of magnesium ion in the state of GTP. In fact, however, calcium ion has another effect of dissociation on TC-GTP in the presence of magnesium. These facts support the results indicated previously[14] that calcium ion has two kinds of roles in microtubule assembly and that magnesium is necessary for calcium to inhibit microtubule formation.

As shown previously, TC-GTP has two classes of binding sites of calcium ion[14].

We believe that the inhibition effect of calcium ion was due to the high-affinity site of calcium in the protein. Even if magnesium ion is present in the polymerization system, TC-GTP has a calcium-binding activity. It is believed that this calcium-binding activity in the presence of magnesium is due to the high-affinity site because the low-affinity sites disappeared in the presence of magnesium in the Scatchard plot of the calcium-binding experiment, while two classes of affinity sites were observed in the absence of magnesium. The results indicated in this paper support the previous proposal. In the case of GTP, calcium is able to bind to the tubulin-colchicine complex and indicates a small association effect similar to that of other metal ions in the absence of magnesium, while calcium ion has a dissociation effect, which induces the inhibition effect of polymerization in the presence of magnesium.

This means that the presence of magnesium and the GTP state are necessary for the inhibition effect of calcium on polymerization. The extrapolated value of the sedimentation coefficient of TC-GDP indicated the α-β dimer state of a protein molecule[14]. In the absence of magnesium and calcium, the ultracentrifugal behavior of the tubulin-colchicine complex is the same between the GTP and GDP states. However, the effect of the salt concentration on the complex of tubulin and colchicines differs significantly. In the absence of magnesium, the addition of calcium made a curve in the concentration dependence of the sedimentation coefficient of TC-GDP, while a least squares-fitted straight line was obtained in the concentration dependence of TC-GDP. In the case of TC-GTP, the concentration dependence was applied to a least squares fit even when 2 mM $MgCl_2$ was present in the solution. The comparison of the results in this paper with our previous ones[14] indicates the molecular shapes of tubulin and the tubulin-colchicine complex changed according to the hydrolysis of GTP to GDP and that the sizes of Tu-GDP and TC-GDP were smaller than those of Tu-GTP and TC-GTP, respectively. The rates of microtubule formation from the tubulin and polymerization from the tubulin complex were not in proportion to the rate of GTP hydrolysis[26,30,31] when the rate of microtubule formation and polymerization were measured by the increase of turbidity. We certainly do not believe that there is only one reason. However, one reason could be that the shape of the microtubule or polymer shrinks after formation of the microtubule or polymerization with the hydrolysis of GTP to GDP.

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Communicated by Takii Yukio