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Interaction of Tubulin with Myosin. IV.[1] Inhibition of Actomyosin Adenosine 5′-Triphosphatase Activity by Heat-treated Tubulin

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Tubulin subjected to heat, urea, or guanidine hydrochloride treatment differed in some respects from native tubulin, which inhibits actomyosin Mg-adenosine triphosphatase (ATPase) activity and the corresponding turbidity change. Whereas native tubulin inhibited 65% of actomyosin Mg-ATPase activity, tubulin heated at 65—70°C for 3 min reduced the ATPase activity to 8% of the original level. The effective inhibition by tubulin was gradually diminished when tubulin was treated above 75°C. Since such treated tubulin had little effect on myosin Mg-ATPase activity, it seems to inhibit the actin activation through its binding to myosin. The changes of turbidity inhibition of actomyosin by tubulins were similar to those in ATPase inhibition. Heat treatment converted the conformation of tubulin from α-helix to β-structure, and heat-treated tubulin was easily degraded by trypsin. Tubulin denatured with urea or guanidine hydrochloride showed less ATPase inhibition than heat-treated tubulin.

Keywords——tubulin; denaturation; interaction; actomyosin; ATPase; conformation

It has been reported that there are two kinds of interaction between microtubule proteins and actomyosin. One is the interaction between myosin and tubulin and the other is that between actin and microtubule-associated proteins.[2] On the interaction of tubulin with myosin, Hayashi *et al.*[2d] observed by electron microscopy that the complex of tubulin and myosin was formed by side-by-side aggregation of myosin filaments whose cross-bridges were decorated with tubulin particles. The molar ratio of tubulin to myosin was calculated to be about 2 by coprecipitation assay.[2b,e] Tubulin was reported to inhibit actin-activated myosin Mg-adenosine triphosphatase (ATPase) activity and the corresponding turbidity change through its binding to myosin filaments.[2b]

The present study compares the properties of tubulin subjected to heat, urea, or guanidine hydrochloride treatment with those of native tubulin, especially as regards inhibitory effect on actomyosin Mg–ATPase activity. The structural difference between native and heat-treated tubulin was also examined.

Experimental

Preparation of Proteins——Porcine brain microtubule proteins were prepared by a modification of the method of Shelanski *et al.*[3] Tubulin was isolated from a microtubule preparation by phosphocellulose (Whatman P II) column chromatography as described previously.[2b,4] The purified tubulin was dialyzed against 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–KOH (pH 6.5), 1 mM Mg $(CH_3COO)_2$, and 25% glycerol, and stored at −80°C. Myosin was prepared from rabbit skeletal muscle by Perry's method[5] and actin by the method of Spudich and Watt.[6] Myosin and actin were dialyzed against 20 mM MES–KOH (pH 6.5) containing 0.5 M KCl and against 100 mM MES–KOH (pH 6.5), respectively, and used within 10 d. Myosin, actin, and tubulin in this experiment were essentially pure as determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.[7]

Heat treatment of tubulin was conducted by incubation at the desired temperature in 100 mM MES–KOH (pH 6.5), 1 mM Mg $(CH_3COO)_2$, and 25% glycerol.

Assay of Actomyosin ATPase——ATPase activity was determined by measuring the Pi liberation. The reaction mixture consisted of 50 mM MES–KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, 2 mM ATP, 5% glycerol,

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0.1 mg/ml of myosin, 0.05 mg/ml of actin, and 0.2 mg/ml of tubulin in a final volume of 0.4 ml. The reaction was started by adding ATP and stopped after incubation for 3 to 5 min at 37°C by mixing 0.4 ml of 20% trichloroacetic acid. Inorganic phosphate was measured by the method of Martin and Doty.[8]

Measurement of Turbidity——Turbidity was monitored by measuring the change in absorbance at 660 nm with a recording spectrophotometer (Japan Spectroscopic Co., Ltd., UVIDEC-410) as described previously.[2b] The reaction mixture contained 0.24 mg myosin, 0.12 mg actin, and 0.08 mg tubulin in 1 ml of a buffer solution containing 80 mM MES–KOH (pH 6.5), 2 mM $Mg(CH_3COO)_2$, 5% glycerol, and 2 mM ATP.

Circular Dichroism (CD)——CD spectra were measured with a Jasco J-40 automatic recording dichrograph at room temperature, approximately 23°C. The CD measurement was performed in a 0.1 mm cylindrical quartz cell. The CD data are expressed in terms of mean residue ellipticity, $[\theta]$. The mean residue molecular weight of tubulin was assumed to be 115 from the amino acid composition.[9]

Protein Determination——Protein was determined by the method of Lowry *et al.*[10] with bovine serum albumin as a standard.

Results and Discussion

Effects of Heat-treated Tubulin on Actomyosin Mg-ATPase

Tubulin, a main component of microtubules, inhibits actomyosin Mg–ATPase activity, but the order of addition of myosin, actin, tubulin, and ATP to the reaction mixture alters the extent of the ATPase inhibition.[2b,11] Therefore, the reaction was started by adding ATP after mixing actin with a buffer containing myosin and tubulin in the present experiment.

Treatment of tubulin at various temperatures (from 37 to 90°C) for 3 min resulted in enhancement of the inhibitory action on actomyosin Mg–ATPase activity as shown in Fig. 1. The inhibitory effect of tubulin increased with increasing temperature of heat treatment in the range of 37—70°C, and maximal inhibition was observed when tubulin was heated at 65—70°C. Figure 2 shows the effect of time of heat treatment of tubulin at 65°C on actomyosin Mg–ATPase activity. After 30 s, tubulin exhibited a potent inhibitory action on actomyosin Mg–ATPase activity and the inhibition was gradually reduced with longer periods of heat treatment at 65°C. Tubulin showed the most effective inhibition of the Mg–ATPase activity when it was heated at 65—70°C for about 3 min. The maximal inhibition reached 25% of actomyosin Mg–ATPase activity in the presence of untreated tubulin and this value corresponded to only 8% of the original ATPase activity. The extent of inhibition was close to that produced by a mixture of troponin-I and tropomyosin from skeletal muscle.[12]

The inhibition of actomyosin Mg–ATPase activity is illustrated as a function of the concentration of tubulin in Fig. 3. The concentration of heat-treated tubulin which was required for half-maximal inhibition of the Mg–ATPase activity was 0.032 mg/ml, whereas that of native tubulin was 0.035 mg/ml. The inhibition reached a plateau when over 0.1 mg/ml of heat-treated tubulin was added to the reaction mixture containing 0.1 mg/ml of myosin and 0.05 mg/ml of actin.

Tubulin was reported to have no effect on myosin Mg–ATPase activity,[2d] and this finding was confirmed in our experiment. Tubulin which was treated for 3 min at 65°C also had little effect on myosin Mg–ATPase activity (data not shown). Therefore, heat-treated tubulin was considered to inhibit actin activation of myosin Mg–ATPase activity.

Effect of Denaturation Reagents on Tubulin

Tubulin which was dialyzed against 100 mM MES–KOH (pH 6.5) and 25% glycerol after being mixed with various concentrations of urea or guanidine hydrochloride also showed enhanced inhibitory action on actomyosin Mg–ATPase activity. The results are summarized in Table I. Tubulin treated with 2 M guanidine hydrochloride failed to show enhanced inhibitory action. On the other hand, tubulin treated with urea (4 and 6 M) showed potent inhibition of the Mg–ATPase activity. However, the inhibition did not reach the level produced by using the heat-treated tubulin.

Effects of Heat-treated Tubulin on Turbidity Change of Actomyosin induced by Mg-ATP

Turbidity increase of actomyosin by Mg–ATP (superprecipitation) is closely related to the

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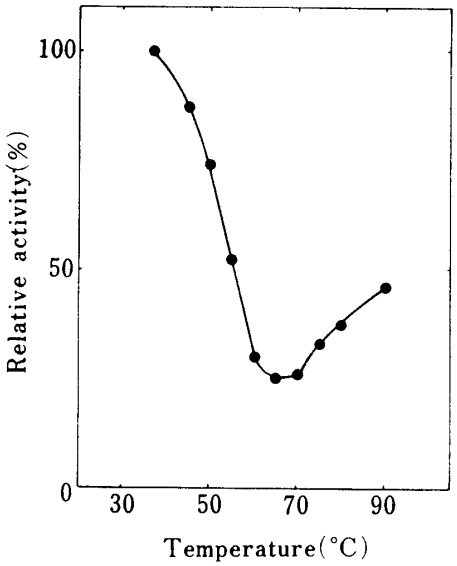

Fig. 1. Effects of Heat-treated Tubulin on Actomyosin Mg–ATPase Activity

Tubulin (2 mg/ml) in a buffer containing 100 mM MES–KOH (pH 6.5), 1 mM Mg $(CH_3COO)_2$, and 25% glycerol was heated at the indicated temperature for 3 min then rapidly cooled. Experimental conditions were as described in Experimental. Actomyosin Mg–ATPase activity in the presence of native tubulin was taken as 100%. Specific activities of actomyosin Mg–ATPase in the presence and absence of native tubulin were 160—190 and 450—530 nmol Pi/min/mg of myosin, respectively.

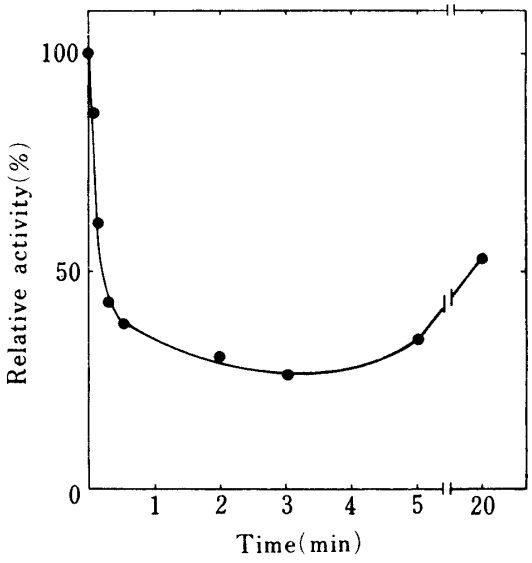

Fig. 2. Effects of Time of Heat Treatment of Tubulin at 65°C on Actomyosin Mg–ATPase Activity

Experimental conditions were the same as in Fig. 1.

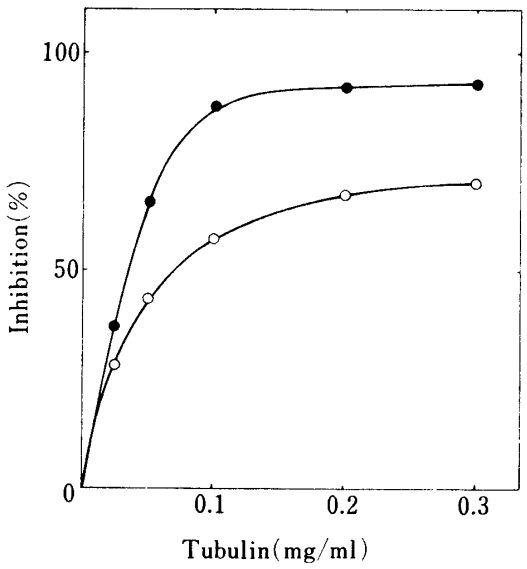

Fig. 3. Inhibition of Actomyosin Mg–ATPase Activity by Native and Heat-treated Tubulin

Heat treatment was performed by incubation of tubulin for 3 min at 65°C. ○, native tubulin; ●, heat-treated tubulin.

actin-activated Mg–ATPase activity of myosin, and tubulin also inhibits the turbidity increase.[2b,11] Therefore, heat-treated tubulin was compared with native tubulin with respect to the inhibitory action on the turbidity increase. As shown in Fig. 4, tubulin which had been treated for 3 min at 65°C showed more potent inhibition of the turbidity change (Δ 660 nm) than untreated tubulin.

Effect of Heat Treatment on the Conformation of Tubulin

Eipper reported that purified rat brain tubulin containing 1 mol of guanine nucleotide per mol of dimer would have an absorbance ratio (A_{280}/A_{260}) of 1.51 calculated on the basis of the molar extinction coefficient, and the values in 6 M guanidine hydrochloride and 0.3% SDS were 1.65±0.03 and 1.67±0.04, respectively.[13] The ratio of A_{280}/A_{260} in native tubulin was 1.49, which is very close to the reported value, and the ratio in heat-treated tubulin decreased to 1.32, different from those of denatured tubulins reported by Eipper. These

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Table I. Effects of Tubulins denatured with Urea and Guanidine Hydrochloride on Actomyosin Mg-ATPase Activity

Treatment	Concentration (M)	ATPase activity (%)
None		100
Urea	2	84
	4	54
	6	50
Guanidine hydrochloride	0.5	77
	1	80
	2	155

Experimental conditions were as described in "Experimental". Actomyosin Mg-ATPase activity in the presence of native tubulin was taken as 100%.

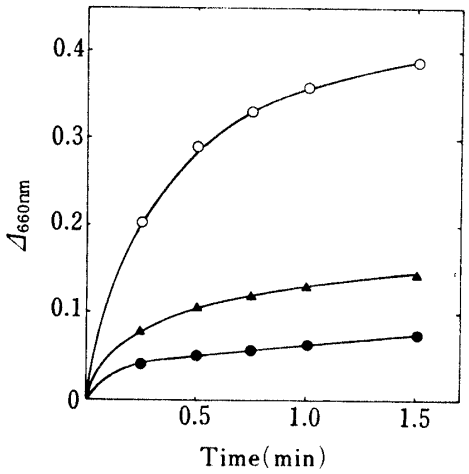

Fig. 4. Effects of Tubulin on the Turbidity Change of Actomyosin induced by Mg-ATP

The order of addition of proteins was the same as in the assay of ATPase activity and the incubation conditions were those given in "Experimental". Heat-treated tubulin was prepared as described in Fig. 3. ○, (−) tubulin; ▲, native tubulin; ●, heat-treated tubulin.

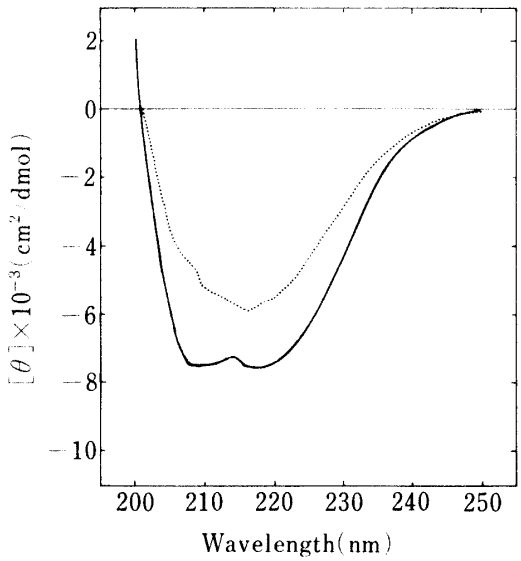

Fig. 5. CD Spectra of Native and Heat-treated Tubulins in the Ultraviolet Region

The concentration of tubulin was 1 mg/ml and the solvent was 100 mM MES-KOH (pH 6.5), 1 mM Mg $(CH_3COO)_2$, and 25% glycerol. Heat-treated tubulin was prepared as described in Fig. 3. ——, native tubulin; ----, heat-treated tubulin.

results suggested that the heat treatment might lead to some conformational change of tubulin. Therefore, the conformation of tubulin was investigated by measuring the CD spectrum in the ultraviolet region. Native tubulin had two negative bands at 208 nm and 222 nm, which are typical of α-helical structure. These negative bands decreased in magnitude with increasing temperature of heat treatment. Tubulin heated for 3 min at 65°C (which showed maximal actomyosin Mg-ATPase inhibition) had only one negative band at 217 nm, indicating the presence of β-structure (Fig. 5). Therefore, heat treatment decreased the α-helix content of tubulin molecule and increased the β-structure content.

In a preliminary study, 2,4,6-trinitrobenzene 1-sulfonic acid, a reagent for labeling primary amino groups in amino acids and proteins,[14] reacted more with heat-treated tubulin than with native tubulin. The extent of hydrolysis of both native and heat-treated tubulin by trypsin was next examined. The results are presented in Fig. 6. Tubulin consists of subunits with a molecular weight of 55000.[15] When native tubulin was treated by trypsin at 37°C, fragments

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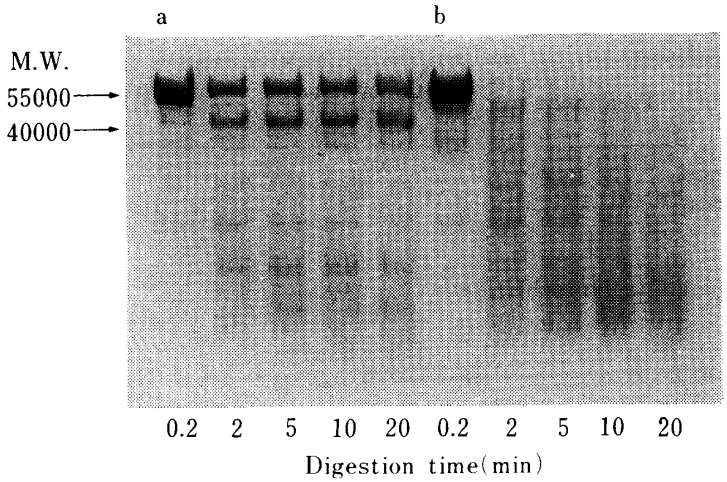

Fig. 6. Electrophoresis of Tubulin digested with Trypsin on 12% Polyacrylamide Gels

Tubulin (1 mg) was digested with 10 μg of trypsin (Worthington) at 37°C in 0.5 ml of 100 mM MES–KOH (pH 6.5), 1 mM Mg $(CH_3COO)_2$, and 25% glycerol. At the specified times, 80 μl aliquots were taken, and mixed with 20 μl of a solution containing 5% SDS and 1% 2-mercaptoethanol, then immersed in boiling water. Heat-treated tubulin was prepared by the same procedure as in Fig. 3.
a: native tubulin; b): heat-treated tubulin.

with high molecular weights of 55000 and 40000 were observed even after 20 min of digestion. On the other hand, the rate of proteolysis of tubulin which had been treated for 3 min at 65°C was more rapid, and after 2 min of digestion with trypsin, the contents of these two fragments with high molecular weight had greatly decreased. This result suggests that some part(s) of the tubulin molecule rich in arginyl and lysyl residues were exposed by the heat treatment, and heat-treated tubulin thereby became more susceptible to trypsin digestion.

It is already known that troponin-I (pI 9.3), F_1-inhibitor (pI 7.6), salmine (pI 12.1), lysozyme (pI 11.1), and cytochrome c (pI 9.8) inhibit actomyosin ATPase.[16] These basic proteins have been considered to act by binding to actin filaments, probably through an ionic interaction, since actin is an acidic protein. Tubulin, although it is a complex of acidic subunits (pI 3.5—5.7),[17] appears to interact with myosin filaments through an ionic interaction, *i.e.*, basic region(s) on the surface of the tubulin molecule interact with myosin filaments. Upon heat treatment, more basic part(s) become exposed, and these newly exposed basic part(s) may strengthen the interaction of tubulin with myosin filaments, leading to an increased effect on enzyme activity and turbidity change.

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References and Notes

1) Part III: T. Fujii, M. Kumasaka, Y. Kondo, A. Hachimori, and K. Ohki, *Chem. Pharm. Bull.*, **29**, 2639 (1981).
2) *a*) L.M. Griffith and T.D. Pollard, *J. Cell Biol.*, **78**, 958 (1978); *b*) T. Fujii and R. Tanaka, *Life Sci.*, **24**, 1683 (1979); *c*) M. Hayashi, *J. Biochem.* (Tokyo), **85**, 691 (1979); *d*) M. Hayashi, K. Ohnishi, and K. Hayashi, *J. Biochem.* (Tokyo), **87**, 1347 (1980); *e*) T. Shimo-Oka, M. Hayashi, and Y. Watanabe, *Biochemistry*, **19**, 4921 (1980); *f*) R.F. Sattilard, W.L. Dentler, and E.L. Lecluyse, *J. Cell Biol.*, **90**, 467 (1981).
3) M.L. Shelanski, F. Gaskin, and C.R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 765 (1973).
4) Y. Ihara, T. Fujii, T. Arai, R. Tanaka, and Y. Kaziro, *J. Biochem.* (Tokyo), **86**, 587 (1979).
5) S.V. Perry, "Methods in Enzymology," Vol. 2, ed. by S.P. Colowick and N.O. Kaplan, Academic Press,

NII-Electronic Library Service

Inc., New York, 1955, pp. 582—588.
6) J.A. Spudich and S. Watt, *J. Biol. Chem.*, **246**, 4866 (1971).
7) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
8) J.B. Martin and D.M. Doty, *Anal. Chem.*, **21**, 965 (1949).
9) M. Ventilla, C.R. Cantor, and M.L. Shelanski, *Biochemistry*, **11**, 1554 (1972).
10) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) K. Ohki, M. Kumasaka, Y. Kondo, and T. Fujii, *Yakugaku Zasshi*, **101**, 848 (1981).
12) M. Shigekawa and Y. Tonomura, *J. Biochem.* (Tokyo), **73**, 1135 (1973).
13) B.A. Eipper, *J. Biol. Chem.*, **249**, 1407 (1974).
14) T. Okuyama and K. Satake, *J. Biochem.* (Tokyo), **47**, 454 (1960).
15) J. Bryan and L. Wilson, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1762 (1971).
16) S.V. Perry, *Symp. Soc. Exp. Biol.*, **27**, 531 (1973); H. Syska, J.M. Wilkinson, R.J.A. Grand, and S.V. Perry, *Biochem. J.*, **153**, 375 (1976); S. Yamazaki, H. Hasebe, H. Takisawa, Y. Tamura, and Y. Inada, *Biochem. Biophys. Res. Commun.*, **75**, 1104 (1977).
17) H.J. George, L. Misra, D.J. Field, and J.C. Lee, *Biochemistry*, **20**, 2402 (1981).

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